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For the degree of Master of Science

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TRANSCRIPTIONAL REGULATION OF RETINAL PROGENITOR CELLS DERIVED FROM HUMAN INDUCED PLURIPOTENT STEM CELLS

A Thesis

Submitted to the Faculty

of

Purdue University

by

Akshayalakshmi Sridhar

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

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Indianapolis, Indiana

This thesis is dedicated to my parents, my extended family and my uncle, Mr. Nageshwaran for believing in me and providing the encouragement to pursue my dreams.

This thesis is also dedicated to my late grandfather, for introducing me to the world of biology.

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SYMBOLS

GENE	Human Protein
GENE	Human Gene
Gene	Mouse protein
Gene	*Mouse gene*
gene	*Xenopus* gene
gene	*Drosophila* gene

ABBREVIATIONS

bHLH	Basic helix-loop-helix protein
BMP	Bone Morphogenetic Protein
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
EBs	Embryoid Bodies
EFTFs	Eye Field Transcription Factors
EGF	Epidermal Growth factor
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
hESCs	Human Embryonic Stem Cells
hiPSCs	Human Induced Pluripotent Stem cells
hPSCs	Human Pluripotent Stem Cells
ICC	Immunocytochemistry
iPS	Induced Pluripotent Stem
KOSR	Knock-Out Replacement Serum
MEF	Mouse Embryonic Fibroblasts
NIM	Neural Differentiation Medium
SHH	Sonic hedgehog
Q-PCR	Quantitative Polymerase Chain Reaction
RDM	Retinal Differentiation Medium
RNA	Ribonucleic acid
RPE	Retinal Pigmented Epithelium
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction

ABSTRACT

Sridhar, Akshayalakshmi. M.S., Purdue University, August 2012. Transcriptional Regulation of Retinal Progenitor Cells Derived from Human Induced Pluripotent Stem Cells. Major Professor: Jason Meyer.

In order to develop effective cures for diseases and decipher disease pathology, the need exists to cultivate a better understanding of human development. Existing studies employ the use of animal models to study and model human development and disease phenotypes but the evolutionary differences between humans and other species slightly limit the applicability of such animal models to effectively recapitulate human development. With the development of human pluripotent stem cells (hPSCs), including Human induced Pluripotent stem cells (hiPSCs) and Human Embryonic Stem cells (hESCs), human development can now be mirrored and recapitulated *in vitro*. These stem cells are pluripotent, that is, they possess the potential to generate any cell type of the body including muscle cells, nerve cells or blood cells. One of the major focuses of this study is to use hiPSCs to better understand and model human retinogenesis. The retina develops within the first three months of human development, hence rendering it inaccessible to investigation via traditional methods. However, with the advent of hiPSCs, retinal cells can be generated in a culture dish and the mechanisms underlying the specification of a retinal fate can be determined. Additionally, in order to use hiPSCs for successful cell replacement therapy, non-xenogeneic conditions need to be employed to allow for fruitful transplantation tests. Hence, another emphasis of this study has been to direct hiPSCs to generate retinal cells under non-xenogeneic conditions to facilitate their use for future translation purposes.

1. INTRODUCTION

1.1 Retina and its function

The human retina is the part of the eye that allows for the ability to visualize objects. The visual information received by the eye is converted into electrical stimuli by the retina, which are then interpreted by the brain to perceive the objects in front of us. As a result, diseases such as retinitis pigmentosa, macular degeneration and cone-rod dystrophy damage the retina, in turn affecting our vision. Upon remaining undiagnosed, these diseases result in severe phenotypes, ultimately leading to blindness. Hence, efforts are being made to understand the development of retina and its functioning to develop successful treatments for these diseases. In order to do so, one needs to have a clear understanding of the structure of the retina and its development.

The retina is derived from central nervous system and is located towards the back of the eye (Figure 1.1A). It is an organized structure composed of six types of cells: photoreceptors consisting of rods and cones, muller glia cells, horizontal cells, bipolar cells, amacrine cells and ganglion cells (Figure 1.1B). These cells are arranged in specific layers, with the photoreceptor layer being the outermost and the ganglion cells are the innermost. In between these two layers lie the inner nuclear layer consisting of horizontal cells, bipolar and the amacrine cells. Intermingled in these layers are the inner plexiform layer and the outer plexiform layer, which help the inner nuclear area form connections with the ganglion cells and the photoreceptors respectively [2, 3]. The photoreceptors are embedded in the Retinal Pigmented Epithelium (RPE), which

supports and provides nourishment to the photoreceptors. The organization of the retina into such layers forms a highly regulated pathway to encode the visual information in the form of synapses which are then carried to the brain [4].

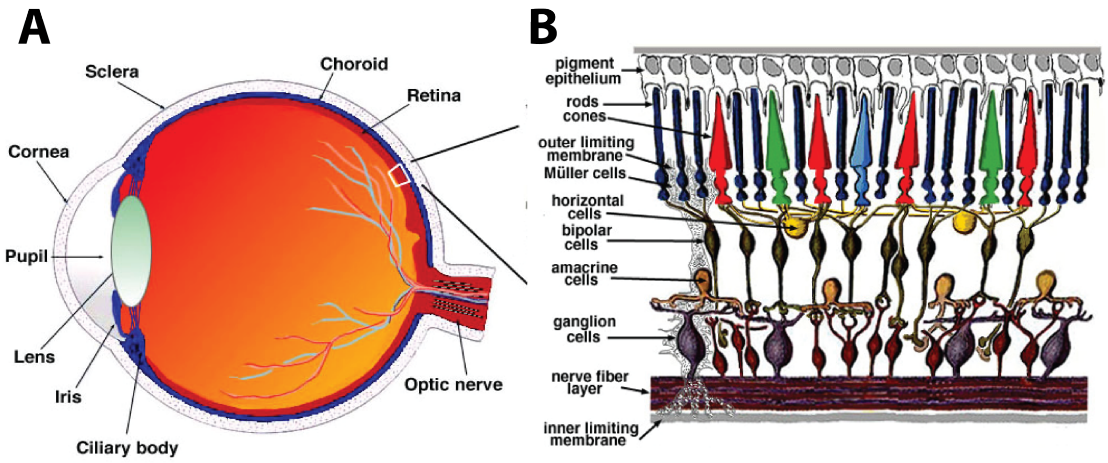

Figure 1.1. [1] Schematic illustration of the human eye and the location of the retina (A). Representation of the different layers of retina (B).

When light reaches the eye, it is focused by the lens and is sent to the back of the eye, where the retina is located. In the retina, photoreceptors convert the visual input to electrical stimuli/neuronal signals which can be processed by the brain. This information is then passed via the bipolar cells to the retinal ganglion cells which carry the information from the eye to specific regions of the brain such as the tectum and the lateral geniculate nucleus via the optic nerve. The results of this pathway help see the object in front of us [5].

Hence, the retina is a highly specialized yet accessible structure which delivers the power of sight. An effort to understand this complex machinery is incomplete without an understanding of how the retina is specified. Therefore, a thorough understanding of the development of the retina is crucial to comprehend its specification.

1.2 Retinal development

The development of the retina is a direct consequence of eye development from the primitive anterior neuroectoderm (Figure 1.2). Eye development is initiated after the process of neurulation, which involves the folding of the neural plate to form the neural tube. As a result of this process, optic grooves form on either side of the developing forebrain. These optic grooves start to evaginate toward the surface ectoderm, forming optic vesicles at the end of the neurulation process. Optic vesicles continue to evaginate until it comes close to the head ectoderm, leading to the induction of head ectoderm to form the lens placode. Retinal specification begins after the formation of lens placode, where the lens placode induces the distal regions of the optic vesicle to form the retina while the surrounding mesenchyme induces the formation of RPE from the proximal regions of the optic vesicle [6, 7]. The spatio-temporal location of the retina and its development is governed by a mixture of growth factors such as SHH (Sonic Hedghog), WNT signaling and Fibroblast Growth Factor (FGF), as well as transcription factors such as *Otx2*, *Pax6*, *Six3*, *Six6* and *Rax* [8].

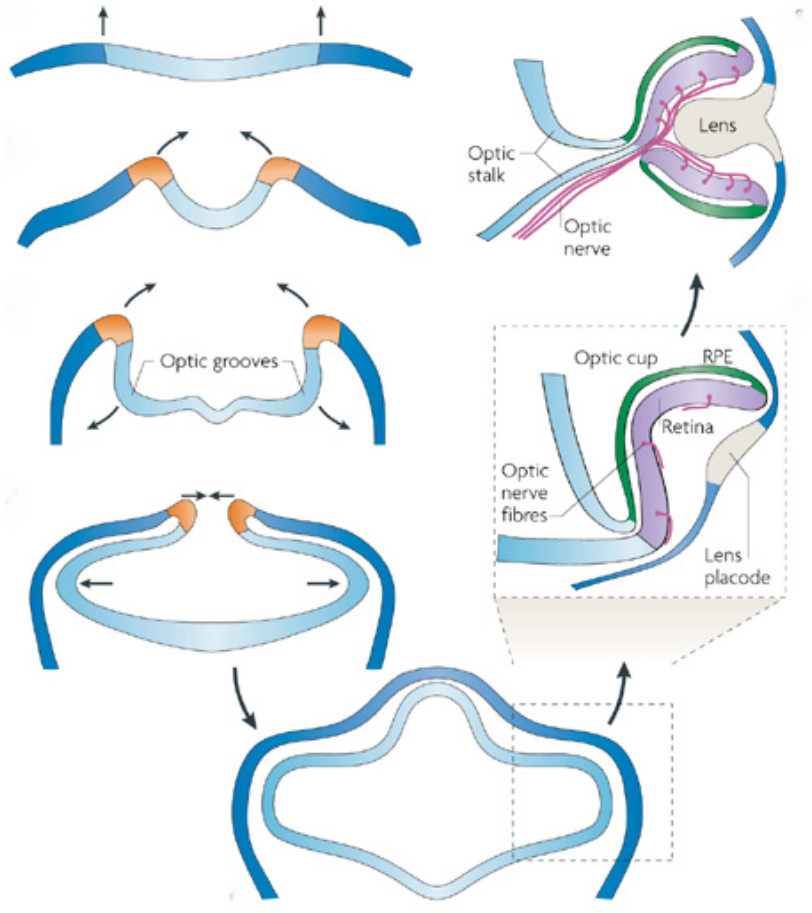

Figure 1.2. [6] Schematic representation of different stages involved in the development of the vertebrate retina.

In general, the specification of different cell types in the nervous system, including the neural retina, is facilitated by both extrinsic and intrinsic factors [9]. Some studies have indicated that the specification of different cell types is governed by competence model, which suggests that different cell types arise depending on the competence stage of the progenitor cell [10]. With respect to the retina, differentiation of these retinal cell types is thought to be accomplished by a combination of basic helix-loop-elix (bHLH) factors such as *Math5* and *Neuro-D*, families of transcription factors, as well as homeobox genes such as *Pax6* and *Six3* [11]. These homeobox genes, along with other transcription factors which were reported to play a major

role in eye development, were grouped together and are collectively known as Eye Field Transcription Factors (EFTFs) [12, 13]. These factors are expressed in specific patterns within the anterior neural plate at very early stages and play an important role in the development and specification of the eye.

1.3 Eye field transcription factors

EFTFs comprise of a group of seven transcription factors, namely *pax6*, *rax*, *six3*, *six6*, *nr2e1(tll)*, *lhx2* and *tbx3(et)* [12,14]. Most of the EFTFs were first discovered in *Drosophila* and previous studies have indicated that these EFTFs highly conserved among different species [15]. They function via a series of multifaceted and reciprocal process of signaling in order to specify the timing as well as the location of the eye during its development. Previous studies performed in model systems such as *Xenopus* have helped to shed light on the highly dynamic and overlapping patterns of expression of these EFTFs [12]. These EFTFs are expressed in a highly coordinated manner in the developing anterior neural plate of *Xenopus* (Figure 1.3). Comparison of the expression patterns of EFTFs at stage 12.5/13 and stage 15 of *Xenopus* development indicates the expression patterns of EFTFs as they trace the eye field upon separation from the neural plate.

The expression of EFTFs is preceded by the expression of a forebrain and midbrain transcription factor *otx2*. Initially, *otx2* is expressed throughout the primitive anterior neuroepithelium [16, 17]. However, following neurulation, *otx2* expression is downregulated towards the epicenter and a void is created, making way for the expression of the EFTFs [12]. It has been demonstrated *OTX2* expression is restricted to optic vesicle and RPE at later stages of the developing chick embryo [18]. Studies

have suggested that the downregulation of *otx2* is triggered by the activation of *rax*, a transcription factor which is also a part of the EFTF network, suggesting the establishment of the eye field in this region [155]. At this stage, the EFTFs are thought to activate each other and function in a coordinated and overlapping manner leading to the development of eyes in this region.

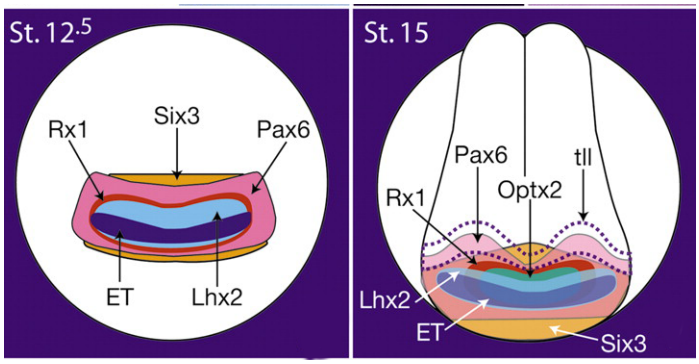

Figure 1.3. [12] Schematic illustration of the overlapping patterns of expression of EFTFs in *Xenopus* at two different stages of its development.

Studies have indicated the expression of EFTFs is necessary and sufficient for retinal fate specification. Injection of these EFTFs (minus *lhx2*) in the two cell stage of the *Xenopus* embryo led to the endogenous activation of *lhx2* and development of ectopic eyes [12]. This suggests that these EFTFs activate each other and function in a very complex, yet remarkably synchronized manner to induce eyes. *Pax6*, which is a part of the EFTF network has been demonstrated to be a fate determinant for the neuroectoderm and also plays a prominent role in determining the phenotypic fate of retinal progenitor cells [20, 21]. Furthermore, misexpression of *pax6* has been shown to generate ectopic eyes in *Drosophila* and *Xenopus* [22, 23].

Overexpression of EFTFs including *Pax6, Rax, Six3* and *Six6* in lower vertebrate systems have led to the expansion of the retinal territory, generation of large eyes or the formation of ectopic RPE [24–28]. Deletion or knockouts of EFTFs like *Rax*

and *Lhx2* in mouse have been known to cause anopthalmia or a lack of eye formation [26, 29]. Malformations in the eyes have been observed in Drosophila owing to mutations of *pax6, six3, six6* [30]. Also, mutations in *Lhx2, Pax6, Nr2e1* and *Rax* in lower model systems have produced severe phenotypes with abnormal eyes or no eyes at all. [26,29,31,32]. Hence, each EFTF has been reputed to play a significant role in the development of the eye. The roles of these EFTFs might also be linked to their pattern of expression; some EFTFs such as *pax6, rax* and *et* are expressed earlier in eye development where as some other factors such as *tll* and *six6* are expressed later [12,13]. This further illustrates the governance of the timing of eye development by EFTFs.

In all, these studies help establish the essential role of EFTFs in development of the eye. However, the precise role of EFTFs in specifying a retinal fate has not been studied in detail. Secondly, though their patterns of expression have been well characterized in lower model systems such as *Xenopus* and mouse, studies related to the characterization of EFTFs in humans and their role in the retinal specification have been largely non-existent due to a lack of an appropriate model.

1.4 Limitation of retinal developmental studies in humans

Studies related to retinal development in humans have been limited due to two main reasons: First, athough animal models such as *Xenopus*, zebrafish and mouse have provided a foundation upon whch we have been better able to understand human development, the evolutionary differences between humans and other vertebrates may occasionly limit the applicability of these studies to be translated to a human system [33]. For instance, the development of a mouse from an embryo takes only three weeks whereas humans have a long gestation period of nine months [34–36]. Also, though the homeobox genes have been conserved across most species, their patterns of expression differ from one species to another. For instance, in mouse, *Sox1* is

expressed before *Pax6* whereas in humans, the reverse order is followed [21]. Furthermore, animal models have certain limitations in their ability to model human diseases. Studies have suggested using rat models to model diseases like macular degeneration [37,38]. However, mice and other lower vertebrates do not possess macula in the eye, which affects their ability to effectively serve as a model system to map diseases like age related macular degeneration [39, 40]. Furthermore, rodents have been demonstrated to possess a higher ratio of rods to cones than primate counterparts, thus underscoring further differences between these models and humans [41].

Secondly, eye development occurs very early in humans, specifically, during the first trimester of human embryogenesis [42, 43]. This raises significant ethical issues for isolating and culturing the embryos at this stage. Some labs have cultured adult fetal tissues and expanded them in vitro to form photoreceptor cells. However, only 30% of the cells from this culture system exhibited a photoreceptor like morphology [44]. Also, these cells do not possess the innate ability to be cultured indefinitely without changes in the differentiation ability of these cells. Furthermore, from a human fetal source, cellular material tends to be highly limited in abundance.

Hence, the need exists to develop a model system that not only recapitulates early developmental events in humans that would otherwise be inaccessible to investigation but also offers distinct technical advantages when compared to traditional methods. The advent of human pluripotent stem cells (hPSCs), including both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), helps overcome some of the disadvantages associated with traditional model systems such as *Xenopus* or mouse and hence, serve as powerful *in vitro* models for studies of human development and disease.

1.5 Human induced Pluripotent Stem Cells (hiPSCs)

Stem cells have always received attention from the medical field due to their ability to self-renew and differentiate into cell types of various lineages such as blood cell, nerve cells or muscle cells [45]. In humans, adult stem cells are found in limited areas of the body such as mesenchymal stem cells of the bone marrow or neural stem cells of the hippocampus [46]. It has been demonstrated that these cells are multipotent and can generate cells of their particular cellular lineage. The restricted self-renewal capabilities of these cells coupled with the small sample number of these cells *in vivo* have encouraged scientists to look for other potential sources of stem cells. The umbical cord and amniotic fluid are other potential sources of stem cells for regenerative medicine but are not pluripotent in nature [47–49]. However, the first major breakthrough was achieved in 1981 when mouse embryos were first cultured on a culture dish [50]. It was shown that pluripotent stem cells could be isolated from these mouse embryos and the term 'embryonic stem cell' was coined [51]. This technique was applied to humans, producing human embryonic stem cell lines using embryos donated from *in vitro* fertilization clinics [52].

An effort to eliminate the embryonic source of these stem cells was realized through the advent of hiPSCs. These cells were described in 2007 by two groups of leading researchers- Shinya Yamanaka's team at Kyoto University in Japan and James Thomson at the University of Wisconsin-Madison [53, 54]. Through the introduction of retroviruses encoding pluripotency-associated genes into human skin fibroblasts, they were able to reprogram fibroblasts into cells resembling hESCs. Since 2007, hiPSCs have been implicated to hold great potential in the field of regenerative medicine [45, 55].

hiPSCs offer several advantages: Firstly, they are pluripotent by definition, meaning that they can give rise to any adult stem cell type of the human body such as muscle cell or blood cells [53, 56]. Secondly, these cells have been derived by reprogramming

adult somatic cells; unlike embryonic stem cells, which were derived from inner cell mass of the blastocyst stage of an embryo [54]. Thirdly, these cells have been demonstrated to recapitulate the blastocyst stage of human development; thus they serve as excellent model to mirror early stages of human development that are otherwise inaccessible to investigation [57]. And lastly, it has been shown that these cells can be directed to differentiate to form different mature cell types like blood cells, neurons or muscle cells [58, 59, 61]. Owing to these unparalleled advantages, hiPSCs were chosen to serve as an *in vitro* model to map human retinogenesis in the Meyer lab. Hence we seek to use hiPSCs as a model to study and decipher the complexities of retinal development.

1.6 hiPSCs and Regenerative medicine

hiPSCs have been implicated to hold great potential for regenerative medicine. The non-embryonic source of hiPSCs has made it possible to generate pluripotent stem cells from almost any cell type of the body. The advent of non-viral reprogramming vectors and the use of non-xenogeneic techniques have helped promote hiPSCs as an important tool in regenerative medicine [60, 164]. As a result, hiPSCs have been implicated to serve as a critical tool for cell replacement [62, 63, 107]. A successful demonstration of this technique was illustrated in a study performed on humanized sickle-cell anemic mouse [64]. iPS cells were derived from an affected mouse and the gene defect was identified and corrected using gene-specific targeting. These corrected iPS cells were then directed to differentiate into hematopoietic progenitors cells, which rescued the sickle-cell phenotype in affected mice upon transplantation. Similar experiments involving transplantation of iPS derived dopaminergic neurons in rats and hiPS derived cardiomyocytes in rodents helped rescue phenotypes associated with Parkinsons disease and defects in cardiac contractile function respectively [65,66].

Hence, hiPSCs offer a wide array of possibilities for disease modeling and generation of patient-specific cell lines. They aid in modeling the disease phenotype *in vitro* and help to identify fundamental differences within the cells of individual patients. This is especially important in diseases like age-related macular degeneration, long QT syndrome or spinal muscular atrophy where evolutionary differences limit disease modeling using animals [67]. One of the earliest applications of this protocol was illustrated in 2008 where iPS cell lines where derived in order to model several diseases including Huntington's disease, Parkinson's disease and Down's Syndrome [68, 69]. One of the best examples of the use of hiPSCs was demonstrated in an attempt to model Spinal muscular atrophy [70]. Fibroblasts were derived from an affected patient and were induced to form iPS cells. These cells were then differentiated to form motor neurons that illustrated the deficits in the disease phenotype when compared to the wild type. Similar experiments performed on other diseases including LEOPARD syndrome, Familial dysautonomia, Long QT syndrome and Gyrate atrophy further illustrate the applicability of diseases to successfully recapitulate the disease phenotype [71–78]. The present use of this technology however is greatly limited to simple monogenetic diseases and needs to be further developed to successfully mirror complex diseases like Alzheimers which have a long latency period [79].

Owing to their innate ability to recapitulate human development *in vitro*, hiPSCs have also been implicated in their role in drug development. However, a number of steps need to be followed to successfully use iPSCs for drug development [80]. First, reliable samples (fibroblasts or blood samples) need to be obtained from a patient with the disease phenotype along with appropriate controls in accordance with the established guidelines [81]. Second, high quality iPS cell lines need to be generated and be characterized extensively. Third, the cell lines should possess the capability to differentiate to the cell types affected in the disease and successfully recapitulate the disease phenotype. Last, an effective and robust assay needs to be generated for the disease phenotype with the capability of conversion into a large-scale assay

for high-throughput screening of drugs in future. One of the first examples of this technique was demonstrated in human embryonic stem cells where potentiaters of glutamate receptors were identified [82].

Despite many advances to date, significant obstacles still remain before this potential is realized due to the possibility of graft rejection and zoonosis resulting from the use of animal products or other undefined components in the medium which need to be addressed before effective cell replacement therapy can be warranted [84, 102]. Hence, a major goal of this field of research is the development of xeno-free differentiated progeny cells derived from hPSCs which could then be successfully used for translational research and regenerative medicine.

1.7 hiPSCs and Retinal development

Human pluripotent stem cells, including Human Embryonic Stem cells (hESCs) and hiPSCs, provide a unique *in vitro* system to study human development. This is particularly useful to study early events in development, such as retinogenesis. Several approaches have been used to model retinogenesis *in vitro* using hESCs: One of the first experiments illustrating the use of hESCs was described in 2004 where hESCs were directed to form RPE [85]. Later, in 2006, it was demonstrated that retinal progenitor cells could be derived using hESCs using three factors namely IGF, Dkk-1 and Noggin [86]. It had been previously demonstrated that the use of Bone Morphogenetic Protein (BMP) and WNT inhibitors is necessary to direct cells to anterior neural phenotype [87–90]. Hence, in 2009, another group of researchers derived retinal cells from hESCs-specifically photoreceptors and RPE utilizing other factors in addition to Dkk-1 such as Lefty-A, Sonic hedgehog (SHH), retinoic acid, activin-A [91]. This study was a clear example of mirroring retinal development in humans where the derivation of retinal cells was established in a step-wise manner wherein transcription factors were used to indicate each stage of development. The use of hiPSCs to

derive retinal cells was demonstrated in 2009 where Dkk-1 and Lefty-A were used to direct hiPSCs to a retinal phenotype [92]. As an effort to identify retinal progenitor cells from hiPSCs early in development, an efficient protocol was also developed in 2009 [93]. This protocol not only recapitulated the exact events of retinogenesis but also presented an elegant and unique way to identify retinal progenitor cells as early as twenty days of differentiation. This was also the first demonstration of derivation retinal cells in culture in the absence of any exogenous signaling factors. Furthermore, this study convincingly described the derivation of retinal ganglion cells, photoreceptors and RPE from retinal progenitor cells derived from hiPSCs with an efficiency of almost 90%.

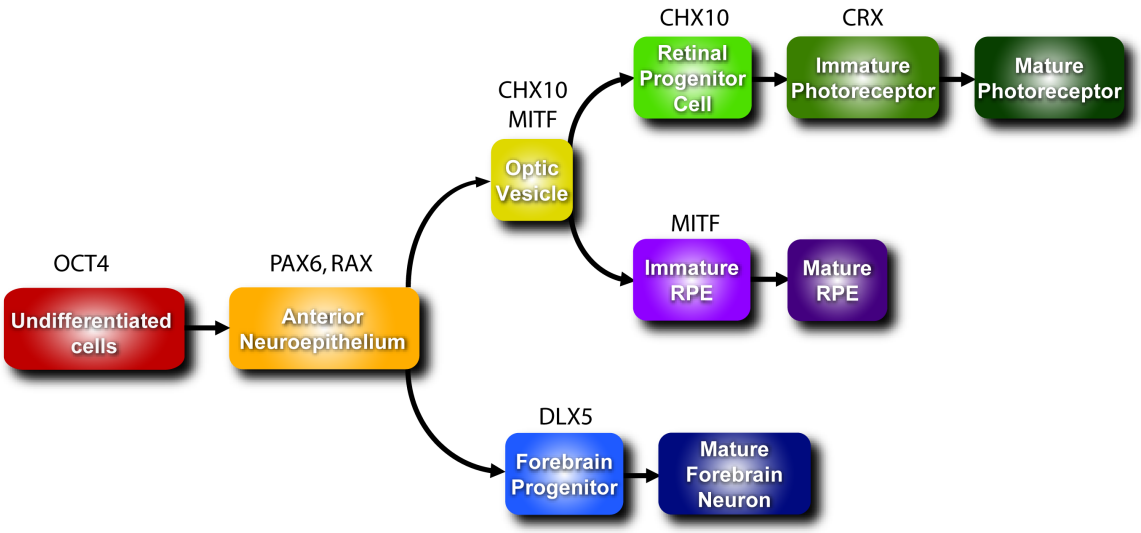

Figure 1.4. [93] Flowchart illustrating the protocol used for directed differentiation of hiPSCs into retinal cells. Markers expressed at different stages of development help model retinogenesis *in vitro*.

It has been previously established that hiPSCs can be directed to cells of an anterior neural lineage in a manner that mimics normal neurogenesis [93, 117] (Figure 1.4). Also, it has been demonstrated that the establishment of a retinal fate from an unspecified stem cell source occurs via a step wise process through an anterior neural

intermediate [91, 93, 105]. The first step in this process is the loss of pluripotency associated markers as the cells progress along the path of differentiation. After ten days of differentiation, a primitive anterior neuroepithelium is established, marked by the coordinated and overlapping expression of the EFTFs [93]. The expression of these EFTFs along with other anterior neural markers provides an anterior specificity to the cells, while laying the groundwork for retinal specification. After 20 days of differentiation, two morphologically distinct populations arise when the cells are in suspension. One type of cell population has a light outer ring surrounding the spherical cells while the other types do not possess the ring-like morphology [117]. These populations express a different set of genes; the cells having the unique phenotype express retina associated genes whereas cells belonging to the other populations express other anterior neural genes of the developing forebrain. The use of morphological features to isolate neurospheres which are reminiscent of the optic vesicle helps in enriching the number of retinal progenitor cells in a mixed population. These spheres then develop into mature retinal cell types such as photoreceptors and ganglion cells over 80 days of differentiation with almost 90% efficiency.

1.8 Aim of the study

hiPSCs have been demonstrated as a unique *in vitro* model with which to better understand human retinogenesis. Although researchers have been successful in deriving retinal cells in culture, the pathways underlying the specification of a retinal phenotype have not been studied in depth. A number of transcription factors and bHLH factors have been known to play an important role in vertebrate retinal development. A close look into these factors suggests prominent roles played by the Eye Field Transcription Factors (EFTFs). Previous studies in lower model systems like *xenopus* and mouse indicate trends in the expression of these EFTFs as the eye develops from the pimitive neuroectoderm [8, 12–14]. For example, certain EFTFs maintain a high level of expression after their onset, while others demonstrate more variable expression

levels [12]. A similar trend is observed when hiPSCs are directed to differentiate into retinal cells where some EFTFS are specifically retained in the retinal neurospheres as opposed to the non-retinal neurospheres, thereby indicating a prominent role of EFTFs in specifying a retinal phenotype [117]. This is interesting as the mechanisms underlying the emergence of these discrete populations of cells in humans have not been studied in depth. Characterization of the trends and roles of EFTFs will help in identifying candidate EFTFs that govern the specification of a retinal fate. Also, a deeper understanding of these mechanisms will help enrich the number of retinal progenitor cells in culture, providing more retinal cells for translational purposes.

To summarize, the experiments described within this thesis seek to use hiPSCs as a unique *in vitro* model with which transcriptional regulation of human retinogenesis can be studied. Additionally, experiments were designed to determine the ability of hiPSCs to be maintained and differentiated into a retinal phenotype under non-xenogeneic conditions for future translational applications.

2. TRANSCRIPTIONAL REGULATION OF RETINAL SPECIFICATION USING hiPSCs AS A MODEL SYSTEM

2.1 Introduction

Development of the vertebrate eye is a complex process that is dependent upon the activity of numerous transcription factors [14, 143]. Out of these factors, the EFTFs have been known to play a crucial role in specifying the timing as well as the location of the eye during its development [12,13]. These include a group of seven transcription factors namely *pax6, lhx2, six3, six6, nr2e1(tll), rax* and *tbx3(et)*. Previous studies done in model systems like *Xenopus* and mouse have helped to shed light on the highly dynamic and overlapping patterns of expression of these EFTFs [8, 14, 95, 96]. However, the evolutionary differences between humans and other vertebrates slightly limit the applicability of these studies to successfully understand human development. Therefore, with the development of Human pluripotent stem cells (hPSCs), including both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), some of the disadvantages associated with traditional model systems such as *Xenopus* or mouse can be overcome and hence, hPSCs serve as powerful *in vitro* models for study of human development.

Human induced pluripotent stem cells (hiPSCs) were first established in 2007 by two groups of leading researchers [53, 54]. Since then, variety of protocols have been developed to direct these cells to a retinal fate [92, 93, 117]. In the Meyer lab, hiPSCs were directed to differentiate toward a retinal lineage using a targeted, stepwise differentiation process that mimics human retinogenesis [93, 116, 117]. From a primitive anterior neural population derived from hiPSCs, populations of retinal and forebrain

progenitor cells could be readily identified within the first 20 days of differentiation. However, the process by which a retinal fate is specified from a primitive anterior neural progenitor cell remains largely elusive. Building upon our previous studies; we sought to establish the role of key transcription factors during the establishment of a retinal fate.

2.2 Methods

2.2.1 Thawing hiPSCs

The hiPS cell line IMR90-4 was chosen by the Meyer lab to study retinal development *in vitro*. This cell line was chosen due to its ability to generate retinal cells and mirror retinogenesis, as demonstrated previously. [93, 117]. The cells were obtained from WiCell and were immediately thawed in a water bath at 37 °C upon delivery. They were then transferred to a 15ml conical tube and spun at 800rpm for one minute on a bench-top centrifuge for removal of DMSO (anti-freeze agent) from the medium. The cells were then re-suspended in 2ml of TeSR medium and were plated on plates coated with matrigel.

2.2.2 Passaging hiPSCs

hiPSCs were supplemented by the addition of 2ml of TeSR medium per well on a daily basis. Upon reaching the desired confluency of 70-80%, good colonies were expanded in a ratio of 1:6. Compact and homogenous colonies with a defined boundary were archetypes of good colonies and were expanded further. Colonies in which individual cells could be visualized were removed manually. hiPS clonies were lifted from the

plate by enzymatic treatment with dispase followed by three washes with DMEM-F12. They were then subjected to vigorous pipetting to disassociate the colonies into smaller clusters and were plated on matrigel-coated plates. Passaging was repeated when hiPSCs reached the desired confluency.

2.2.3 Freezing of hiPSCs

After passaging, cells were re-suspended in medium containing 70% TeSR medium, 20% Fetal Bovine Serum (FBS) and 10% DMSO. Each well accounted for one aliquot; henceforth, six aliquots of one ml each were made from a single six-well plate. These aliquots were stored in a cryovial at -80 °C temporarily, followed by transfer to liquid nitrogen for long-term storage.

2.2.4 Differentiation of hiPSCs

Induction of iPSCs toward an anterior neuroectodermal fate was performed as previously described for hESCs and hiPS cells. Briefly, hiPSCs were enzymatically lifted from 6 well plates using dispase (1mg/ml) with mechanical scraping and grown as aggregates in suspension for 3-4 days to initiate differentiation as embryoid bodies (EBs). The EBs were then switched to a chemically defined neural induction medium (NIM), which consisted of DMEM/F12, N2 supplement, MEM non-essential amino acids and 2mg/ml heparin. Two days later, EBs were allowed to attach with the addition of laminin. Within a few days, columnar cells developed and formed neural tube-like structures within a week. To allow for retinal differentiation, the medium for the hiPSC-derived neuroepithelial rosettes was switched to a retinal differentiation medium (RDM) consisting of DMEM/F12 (3:1) supplemented with B27 on day16 of differentiation. For neural retinal progenitors, rosettes were mechanically isolated from adherent cultures upon change of medium with light trituration. Neurospheres

possessing a light ring-like appearance around the periphery were separated using a P20 pipette. These cells were then placed in suspension culture in non-adherent culture dishes till 20 days of differentiation.

2.2.5 Q-PCR

Cells were removed from the plate with the use of a cell scraper and were collected in 0.5ml tubes. Samples were collected every two days, starting from Day0 (D0) until Day20 (D20) and RNA was isolated from these cells using the RNAeasy kit (Qiagen) and Picopure RNA isolation kit (Life Technologies). During this procedure, a 15 minute DNAase step was done in order to remove any contaminating DNA from the RNA samples. Next, cDNA synthesis was done using iScript cDNA synthesis kit by BioRad. Following cDNA synthesis, the samples were diluted in a ratio of 1:10 and were further analyzed by Q-PCR. For 20μL Q-PCR reactions, 10μL of SYBR green, 4μL of water, 2μL of primers (300μM concentration) and 4μL of DNA were added. Q-PCR was performed for 40 cycles via stages of initial heating (95 °C, 15 minutes), denaturation (95 °C, 20seconds), annealing (60 °C, 30 seconds) and extension (72 °C, 1 minute) stages. Samples were run in triplicates with β-$ACTIN$ as the endogenous control and each experiment was repeated a minimum of three times.

2.2.6 Immunocytochemistry

Neurospheres or embryoid bodies were plated onto poly-ornithine/laminin coated coverslips overnight to allow for attachment. These were then fixed with 4% paraformaldehyde at the required time point in order to examine the translation of the RNA transcripts into mature protein. Following the fixing step, the coverslips were washed three times with PBS followed by incubation with 0.2% triton-X for ten minutes. Non-specific binding of the antibodies was prevented by incubating the cells with 10% donkey serum for an hour. This was followed by the addition of the primary antibody in 5% donkey serum and 0.1% triton-X at the recommended dilution. Fol-

lowing overnight incubation at 4 °C, cells were washed 3X PBS in order to remove the primary antibody. After a brief incubation for 10 minutes in 10% Donkey serum, Secondary antibody and DAPI were added at a dilution of 1:1000. This was followed by 3X washes with PBS and mounting of the coverslips on slides using mounting medium. Labeled cells were visualized with either Cy3-conjugated or Alexafluor488 secondary antibodies and nuclei were stained with DAPI. Images were obtained on a Leica 5500 upright epifluorescence microscope.

Table 2.1
List of antibodies used for Immunocytochemistry

Antibody	Species	Dilution used	Source
ZO-1	Rabbit	1:100	Invitrogen
PAX6	Rabbit	1:100	Stemgent
BESTROPHIN	mouse	1:100	Millipore
β-III TUBULIN	mouse	1:1000	Covance
BRN3	goat	1:200	Santa Cruz Biotech.
CHX10	sheep	1:200	Exalpha Biol Inc.
EZRIN	rabbit	1:100	Cell Signalling Tech.
ISLET-1	mouse	1:200	Exalpha Biol Inc.
LHX2	goat	1:1000	Santa Cruz Biotech.
NANOG	goat	1:20	R&D Sys Inc.
OCT-4	rabbit	1:100	Stemgent
OTX2	goat	1:1000	R&D Sys Inc.
RAX	rabbit	1:200	Millipore
RECOVERIN	rabbit	1:1000	Millipore
SIX6	rabbit	1:200	Sigma-Aldrich
SOX1	goat	1:1000	R&D Sys Inc.
SSEA-4	mouse	1:100	Stemgent
TRA-1-60	mouse	1:100	Stemgent
TRA-1-81	mouse	1:100	Stemgent
ZO-1	rabbit	1:100	Invitrogen

Table 2.2

Primer sequences used for RT-PCR (A to K)

Gene Amplified	Forward Sequence	Reverse Sequence	Size(bp)
BRACHYURY	ACC CAG TTC ATA GCG GTG AC	CAA TTG TCA TGG GAT TGC AG	392
BRN3	GCA AGC AGG CGT TTA GCA TGC C	CTG GGA GAC GAT GTC CAC GGC T	338
CHX10	ATT CAA CGA AGC CCA CTA CCC AGA	ATC CTT GGC TGA CTT GAG GAT GGA	229
CRX	TAT TCT GTC AAC GCC TTG GCC CTA	TGC ATT TAG CCC TCC GGT TCT TGA	253
CTCF	AGC GCT ATG CCC TCA TCC AGC A	ACA GAC AAA AGC CGC AGG GAC G	239
DLX1	CAA CCA GCA AAT GTC TCC TTC TC	CGC ACT TCA CCG CCT TCC	282
DLX2	CTC CCT CAG CTC TCT CCT CA	TGT GTC CAA GTC CAG GCT AA	195
EMX1	AGA CGC AGG TGA AGG TGT GG	CAG GCA GGC AGG CTC TCC	403
EMX2	CAC AGA AAC GGA CAA CAT GG	CTT TAG ACG AGG GTC GCT TG	234
EN1	CCC TGG TTT CTC TGG GAC TT	GCA GTC TGT GGG GTC GTA TT	162
FOXG1	ACT CAA AAC TCG CTG GGC AAC	CGT GGG GGA AAA AGT AAC TGG	226
GAPDH	ACC ACA GTC CAT GCC AT CAC	TCC ACC ACC CTG TTG CTG TA	450
GSX2	CTC GCT CAT CAT CAA GGA CA	AGT GCA GGT GCG AAG TGA C	188
HOXB4	GCA AAG AGC CCG TCG TCT AC	CGT GTC AGG TAG CGG TTG TA	160
KLF4	AGT CCC GCC GCT CCA TTA CCA A	TGC TCG GTC GCA TTT TTG GCA C	316

Table 2.3

Primer sequences used for RT-PCR (L to Z)

Gene Amplified	Forward Sequence	Reverse Sequence	Size(bp)
LHX2	CAA GAT CTC GGA CCG CTA CT	CCG TGG TCA GCA TCT TGT TA	284
LIN28	AGT GGT TCA ACG TGC GCA TGG G	AGG TCC GGT GAC ACG GAT GGA T	203
MITF	TTC ACG AGC GTC CTG TAT GCA GAT	TTG CAA AGC AGG ATC CAT CAA GCC	106
NANOG	CAA AGG CAA ACA ACC CAC TT	TCT GCT GGA GGC TGA GGT AT	158
NR2E1	ATG GCA AAT TCT GTG GCG CTG AAG	GCG CTG ATT TCC CAA GTG CAT TCT	352
OCT4	CGA GCA ATT TGC CAA GCT CCT GAA	TTC GGG CAC TGC AGG AAC AAA TTC	324
OTX2	CAA CAG CAG AAT GGA GGT CA	CTG GGT GGA AAG AGA GAA GC TG	429
PAX6	CGG AGT GAA TCA GCT CGG TG	CCG CTT ATA CTG GGC TAT TTT GC	300
RAX3	GAA TCT CGA AAT CTC AGC CC	CTT CAC TAA TTT GCT CAG GAC	279
SIX3	CGA GCA GAA GAC GCA TTG CTT CAA	CGG CCT TGG CTA TCA TAC ATC ACA	394
SIX6	ATT TGG GAC GGC GAA CAG AAG ACA	ATC CTG GAT GGG CAA CTC AGA TGT	385
SOX1	CAA TGC GGG GAG GAG AAG TC	CTC TGG ACC AAA CTG TGG CG	464
SOX2	CCC CCG GCG GCA ATA GCA	TCG GCG CCG GGG AGA TAC AT	448
TBX2	TGG ACA GTT CAC CAT GGG CCC T	GCC TCC GAA AGT GGG CAT TGG A	235
α-FETOPROTEIN	AGA ACC TGT CAC AAG CTG TG	GAC AGC AAG CTG AGG ATG TC	676
β-III-TUBULIN	TCG GGG CCA AGT TCT GGG AAG T	CCC TGC AGG CAG TCG CAG TTT T	349

Table 2.4

Primer sequences used for Q-PCR

Gene Amplified	Forward Sequence	Reverse Sequence	Size(bp)
β-ACTIN	CCA GTG GTA CGG CCA GAG G	GCG AGA AGA TGA CCC AGA TC	103
BESTROPHin	GGT GTG GTT TGC CAA CCT GTC AAT	TGT TCA TCT CGT TCA GCA GGC TCT	92
CRALBP	TTC AAG GGC TTT ACC ATG CAG CAG	AGT ACC ATG GCT GGT GGA TGA AGT	130
EZRIN	ACC ACC ATG GAT GCA GAG CTG GA	ACA CTT CCC GGA GGC CGA TAG T	284
LHX2	GCA CCA CCA GCT TCG GAC CA	ACC AGA CCT GGA GGA CCC GC	119
NR2E1	CAC AGA AAC CAG TGC AGG GCG T	CTT GCT TGC GGA TGG TGG ACG T	112
OTX2	AGA GCA GCC CTC ACT CGC CA	AGT CGG CCC AAA TCG GGG GT	190
OCT4	GTG GAG GAA GCT GAC AAC AA	ATT CTC CAG GTT GCC TCT CA	120
PAX6	AGT GAA TCA GCT CGG TGG TGT CTT	TGC AGA ATT CGG GAA ATG TCG CAC	120
PEDF	AGA TCT CAG CTG CAA GAT TGC CCA	ATG AAT GAA CTC GGA GGT GAG GCT	127
RAX	AGC GAA ACT GTC AGA GGA GGA ACA	TCA TGC AGC TGG TAC GTG GTG AAA	81
RPE65	TAC CAC AGA AGG TTC ATC CGC ACT	GGG AAA GCA CAG GTG CCA AAT TCT	92
SIX3	CGG AGC CTG TTG CGG GAG TG	ATG CCG CTC GGT CCA ATG GC	81
SIX6	ACC CCT ACG CAG GTG GGC AA	TGA AGT GGC CGC CTT GCT GG	198
SOX1	TCA AAC GGC CCA TGA ACG CCT	TTC TCG GCC TCG GAC ATG ACC T	139
TBX3	AGG CAC GGG AGA AGA GCC CA	AAC CCC CTT CTA CCA GCG CTA TCA A	141
ZO-1	AGA CCG TGC TGA CTT CTG GAG ATT	ACT TTG TTT GAA CAG GCT GAG CGG	101

2.3 Results

2.3.1 Preliminary data

Previous studies from the Meyer lab have demonstrated the ability to derive retinal cell types from hiPSCs following a method that closely recapitulates what is known about human retinogenesis [93, 117]. However, these retinal cells arise in a mixed population which includes other anterior neural cell types. As hiPSCs are directed to differentiate into retinal cell types, two distinct populations arise within the first twenty days of differentiation as the cells are grown in floating aggregates known as neurospheres. These neurospheres can be identified based on their morphology and manually separated into retinal and non-retinal groups. Those neurospheres with a light outer ring around the periphery develop into retinal cells while cells lacking this morphological feature develop into other anterior neural cells, most notably of the forebrain [117].

Based on these previous results, experiments were designed to better understand the emergence of these two distinct populations and decipher the signals that govern the specification of retinal fate versus forebrain fate. Therefore, samples were collected for the first twelve days of differentiation and were analyzed by RT-PCR. Initially, differentiating cultures of hiPSCs were screened for their expression of neural transcription factors and EFTFs (Figure 2.1). Based on these experiments, it was observed that numerous transcription factors displayed similar trends in their expression patterns where their expression began at day 4 of differentiation and was retained until at least day 12 of differentiation. Of note was the observation that certain transcription factors such as *RAX* exhibited varying trends in their expression patterns in the same time-frame, where *RAX* expression seemed to peak by day 8 of differentiation and was subsequently reduced at later timepoints. Based on this preliminary data, we

decided to extend our time point to twenty days of differentiation and the data was quantified using the technique of Q-PCR to get a deeper insight into the role of these EFTFs in retinal development.

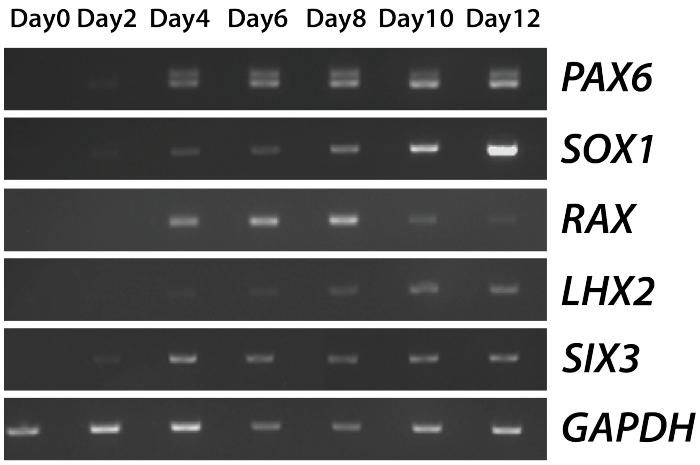

Figure 2.1. RT-PCR data illustrating the expression of EFTFs in hiPSCs over 12 days of differentiation.

2.3.2 Loss of pluripotency

The acquisition of a more differentiated fate from hiPSCs requires that the cells lose characteristics of pluripotency and acquire characteristics of advancing stages of development. Initially, undifferentiated hiPSCs express pluripotency-associated factors in a homogenous and uniform manner. This is illustrated by the techniques of Q-PCR and ICC wherein the cells express markers like *OCT4* and *NANOG* (Figure 2.2). However, upon directing hiPSCs on the route of differentiation, they lose the expression of these genes overtime as demonstrated by immunocytochemistry results, which depict the gradual loss of expression of these markers by day 8 of differentiation (Figure 2.2A,B).

The ICC data was collected until day 8 of differentiation due to lack of a positive signal after that time point while Q-PCR was performed over a timecourse of the first twenty days of differentiation. Q-PCR confirms the earlier trend by establishing the gradual loss of expression of *OCT4* over twenty days of differentiation (Figure 2.2C). *OCT4* expression was maximal at the initiation of differentiation (Day0), with marked decrease in expression at day 2 and day 4. By day 8 of differentiation, *OCT4* expression is minimal and this lack of *OCT4* expression is retained till day 18 of differentiation. Hence, the techniques of ICC and Q-PCR demonstrate the successful of loss of pluripotency-associated genes upon differentiation of hiPSCs.

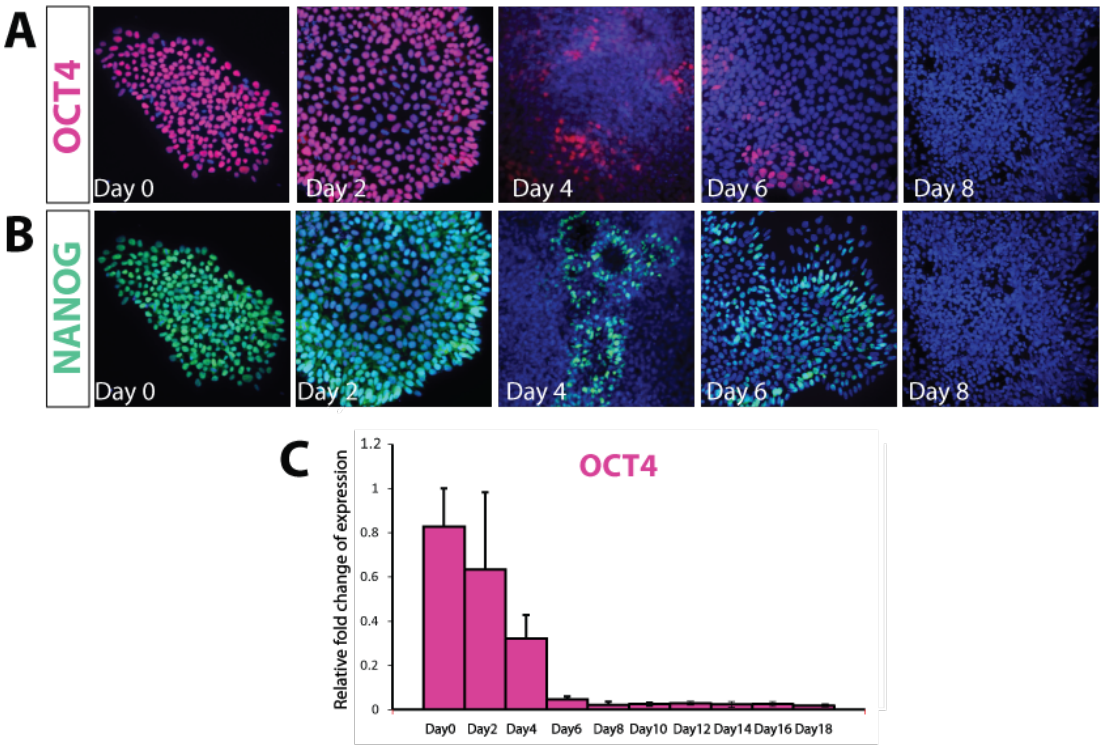

Figure 2.2. hiPSCs lose the expression of pluripotency-associated markers such as *OCt4* and *NANOG* upon differentiation. ICC illustrates the gradual loss of expression of OCT4 (A) and NANOG (B) over 8 days of differentiation. Q-PCR data indicates loss of expression of *OCT4* and *NANOG* over twenty days of differentiation(C).

2.3.3 Primitive anterior neural specification

As hiPSCs are directed to differentiate into cells of the retina, the earliest markers that appear are analogous to the establishment of the anterior neural plate. The expressions of genes such as *PAX6* and *OTX2* at this stage are indicative of the emergence of the nascent anterior neural plate. *PAX6* is a wide-ranging marker of neural progenitor cells while expression of *OTX2* is more confined to the forebrain-midbrain region and their coordinated expression indicates the specification of the primitive anterior neuroepithelium. The eye field is specified from this primitive neuroepithelium at later stages of development.

During the differentiation of hiPSCs to a retinal fate, the expression of these genes occurs very early in development, with the expression of PAX6 beginnings at approximately day 4 of differentiation (Figure 2.3A), while OTX2 expression is observed in undifferentiated cells and is retained as these cells proceed along a neural lineage (Figure 2.3B). As differentiation progresses, the expression of these markers increases till twenty days of differentiation, as demonstrated by immunocytochemistry experiments which depict these patterns of expression of these markers. Q-PCR further recapitulates the trends seen in the expression patterns of *PAX6* and *OTX2* and their role in establishing a primitive anterior neural fate is highlighted (Figure 2.3C,D).

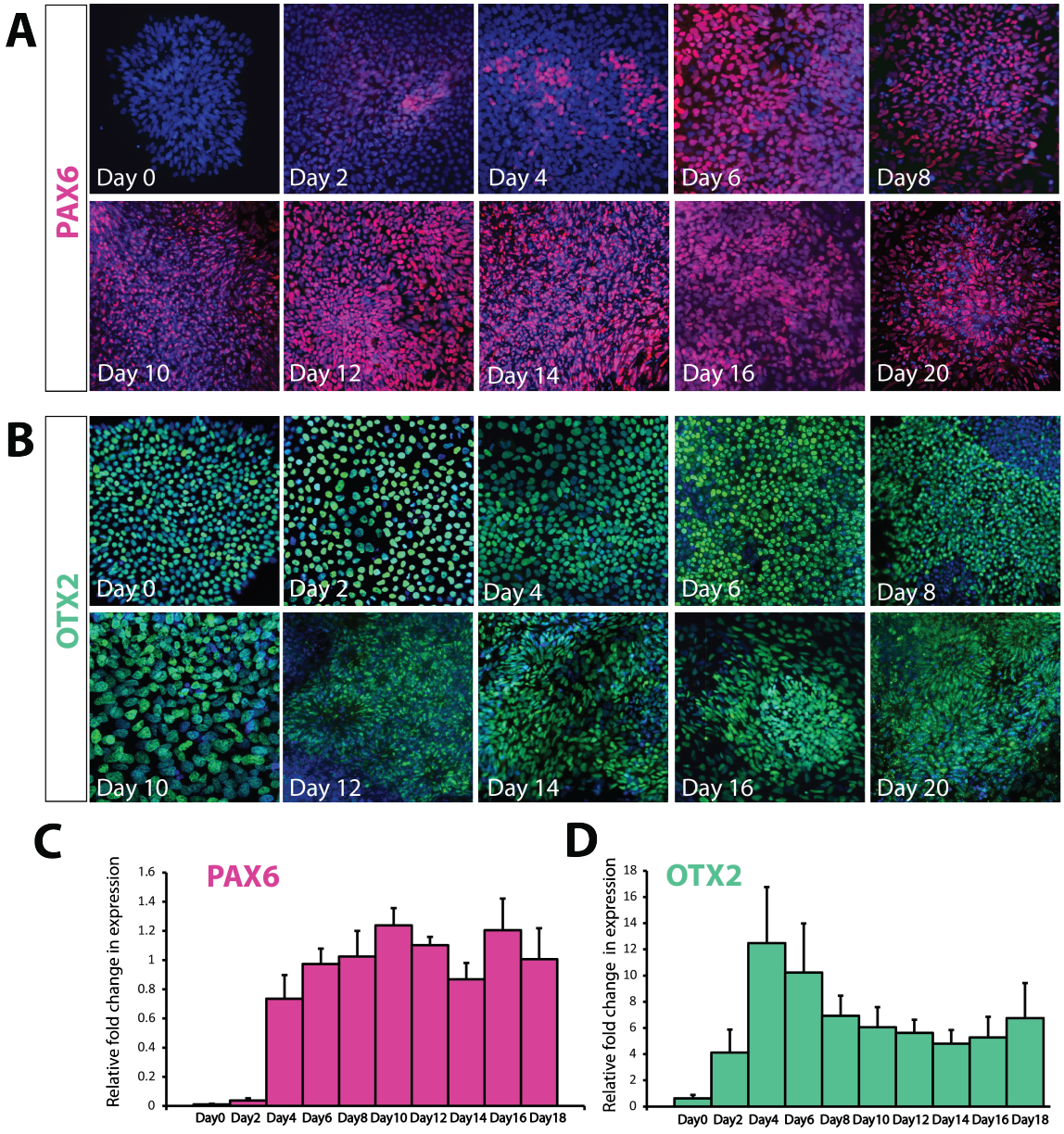

Figure 2.3. The acquisition of a primitive anterior-neural fate was illustrated by the expression of neural transcription factors like *PAX6* and *OTX2* within 4 days of differentiation, with increasing expression levels until 20 days of differentiation (A, B). The trends seen by ICC were quantified by Q-PCR, which further highlights the expression patterns of *PAX6* and *OTX2* over twenty days of differentiation (C, D).

2.3.4 Definitive neural specification

As hiPSCs are directed on the route of differentiation, they mimic neurogenesis by expressing markers specific to different stages of neurulation. The establishment of the anterior neural plate is the first step of neurulation, indicated by the markers *PAX6* and *OTX2*. Next, the neural tube folds on itself to form the neural tube, which is recapitulated in differentiating cultures of hiPSCs by the presence of neural rosettes in culture beginning around Day 12 of differentiation. Cells of the rosettes are positive for transcription factors like *SOX1, PAX6* and *OTX2*, hence establishing a definitive neural identity to these cells. The onset of expression of *SOX1* has been previously demonstrated to correlate with the definitive regional specification of neural cells [94, 152] and thus, the data presented here helps to demonstrate the combinatorial expression of transcription factors including *SOX1, PAX6* and *OTX2*, establishing a definitive neural identity to these cells (Figure 2.4).

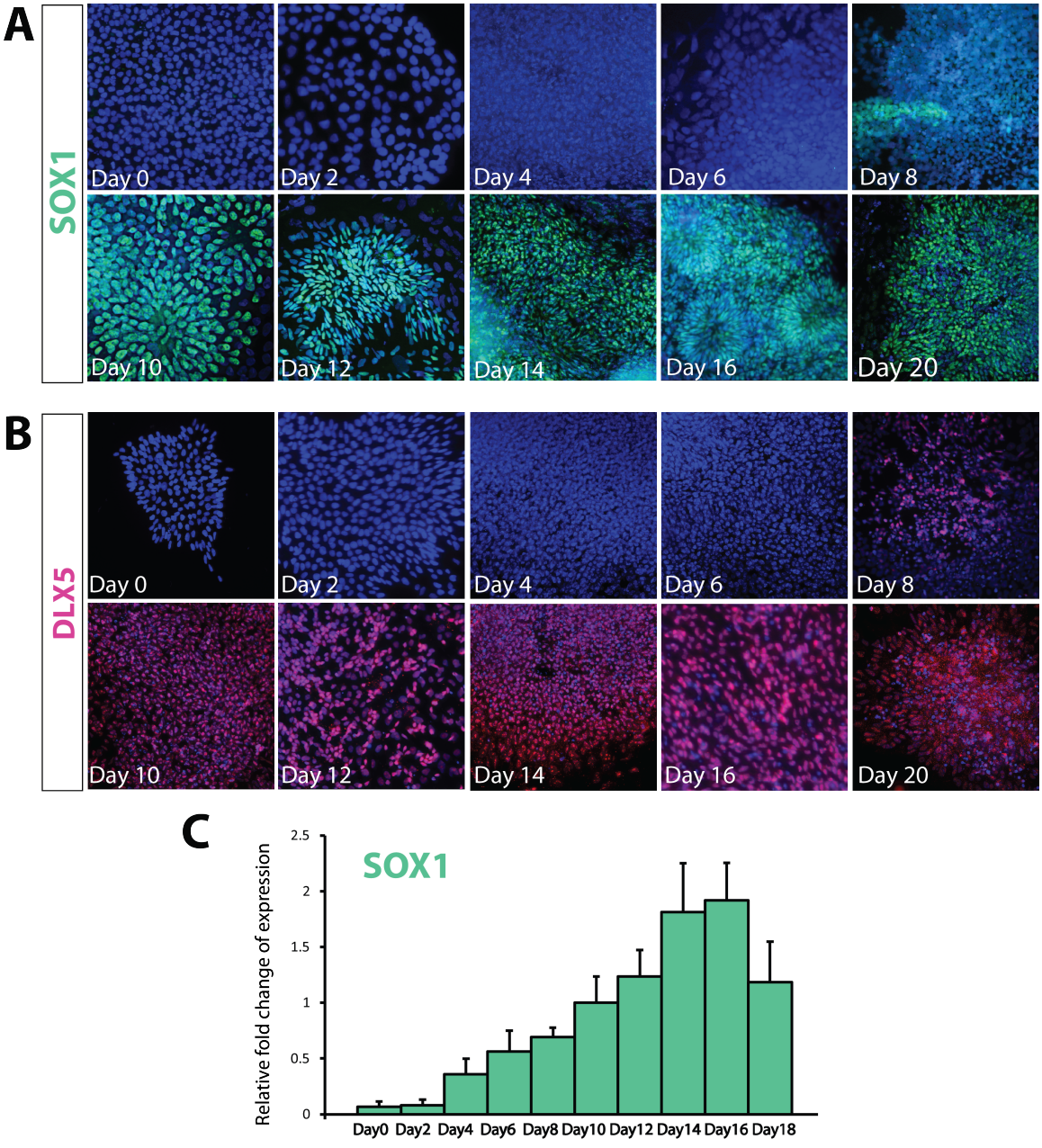

Figure 2.4. Expression of *SOX1* over the first twenty days of differentiation indicated the gradual acquisition of a neural fate (A, C) while expression patterns of*DLX5* (B) over the same time course helped identify a forebrain-specific fate within the differentiating hiPSCs. These markers were differentially expressed in the non-retinal spheres as compared to the retinal spheres [117].

Between the retinal and non-retinal populations of cells derived from this induction protocol, it is likely that important transcriptional differences exist to specify a retinal or forebrain fate. It was observed that the expressions of certain transcription factors were restricted within only a subset of neurospheres, potentially underlying a mechanism by which retinal and forebrain fates are established. Among these transcription factors, *SOX1* is often expressed in many neural progenitor cells, while *DLX5* is more restricted to forebrain regions. Importantly, the expression of these transcription factors is typically not observed in the retina [93]. Q-PCR indicates similar trends in the patterns of expression of *SOX1* and *DLX5*, where their expression starts around day 8 of differentiation and is retained till day 20 of differentiation (Figure 2.4C, D). Immunocytochemistry results further confirms the Q-PCR data wherein the onset of the expression of *SOX1* and *DLX5* is seen at day 8 of differentiation (Figure 2.4A, B). The expression of these factors increases with the progress of differentiation and is retained until at least twenty days of differentiation.

2.3.5 Eye field transcription factors

As the neural plate fold on itself to form the neural tube, the optic grooves are formed on each side develop into optic vesicles upon neurulation. Evagination of optic vesicle towards the head ectoderm causes exchange of signals, leading to induction of the retina and RPE [6]. A number of factors regulate the specification of the retina, with the EFTFs playing a prominent role [14]. EFTFs include a set of transcription factors namely *PAX6, LHX2, SIX3, TBX3, NR2E1, SIX6* and *RAX* [12]. Q-PCR highlights significant trends in the expression patterns of these factors in the differentiating cultures of hiPSCs.

The expression patterns of *PAX6, LHX2, SIX3, TBX3* and *NR2E1* are highly similar, with the onset first evident at approximately day 4 of differentiation and is retained up to at least day 20 of differentiation. Q-PCR confirms the highly coordinated and

overlapping patterns of expression of these factors (Figure 2.5B-E), whereas immunocytochemistry results also reveals trends in the expression patterns of LHX2 similar to what had been observed earlier for *PAX6*, with an onset of expression at day 4 of differentiation and persistent expression until at least day 20 of differentiation (Figure 2.5A). The simultaneous and overlapping expression of these factors is indicative of the complex signaling processes involved in the specification of the eye within the first twenty days of differentiation.

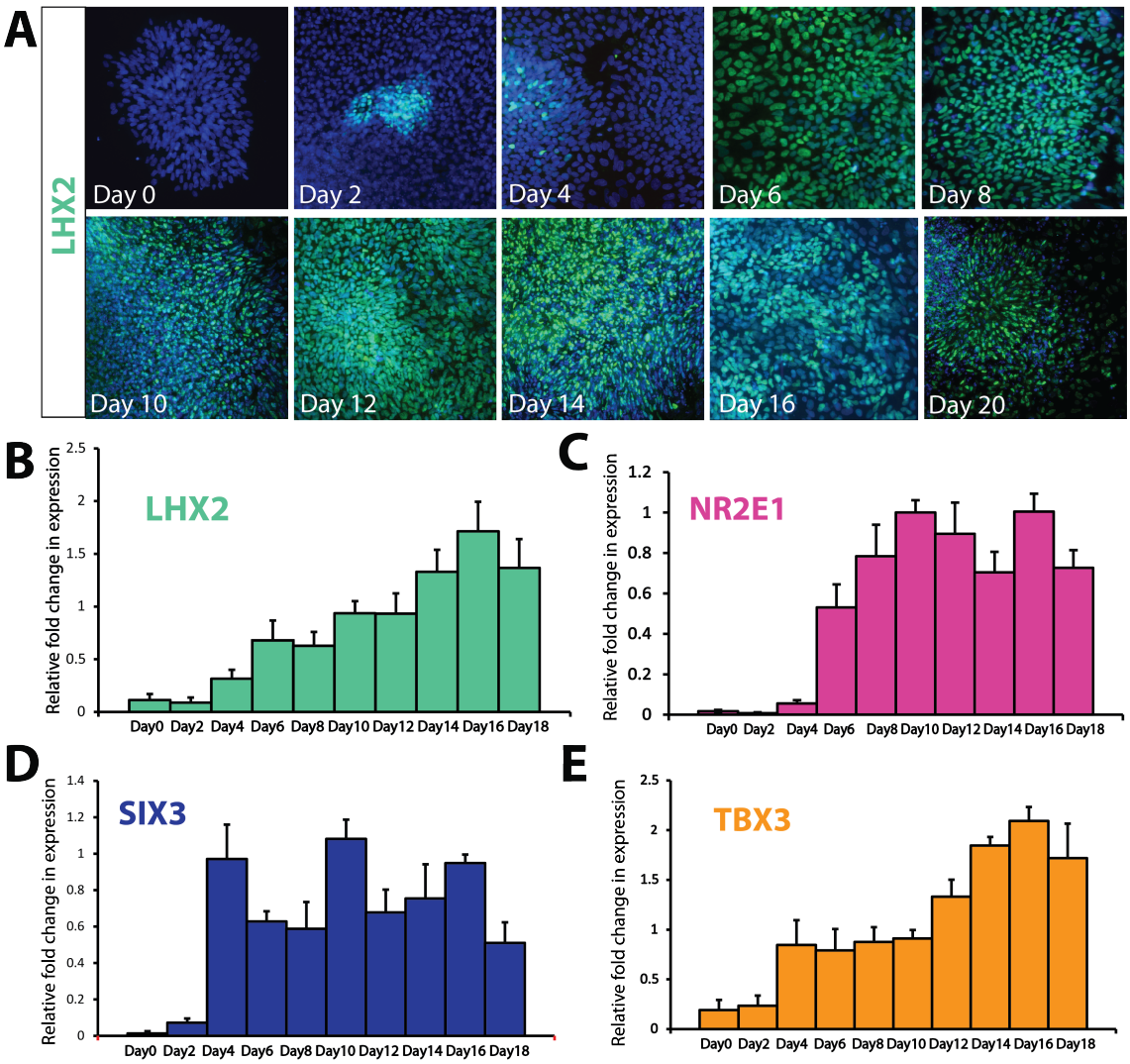

Figure 2.5. Eye field transcription factors (EFTFs) such as *LHX2, TBX3, NR2E1* and *SIX3* displayed similar patterns of expression (A-E). The onset of expression of these genes occurred after approximately 4 days of differentiation and increased through day 20 (B-E). Immunocytochemistry and Q-PCR indicated that the expression of many transcription factors was maintained at high levels, although the expression of *LHX2* began to decrease as observed via immunocytochemistry at some later time points (A,B).

2.3.6 Key retinal specification genes

While many EFTFs were expressed in a ubiquitous fashion, efforts were undertaken to indentify varying patterns of expression that could potentially account for the adoption of a retinal fate. Transcription factors such as *PAX6, LHX2, SIX3, NR2E1* and *TBX3* had very similar patterns of expression, with an onset of expression at day 4 of differentiation followed by an increase of expression until day 20 of differentiation (Figure 2.5). However, *RAX* and *SIX6* were two transcription factors identified that exhibited variations in their expression patterns, potentially indicating a more prominent role in specifying a retinal phenotype.

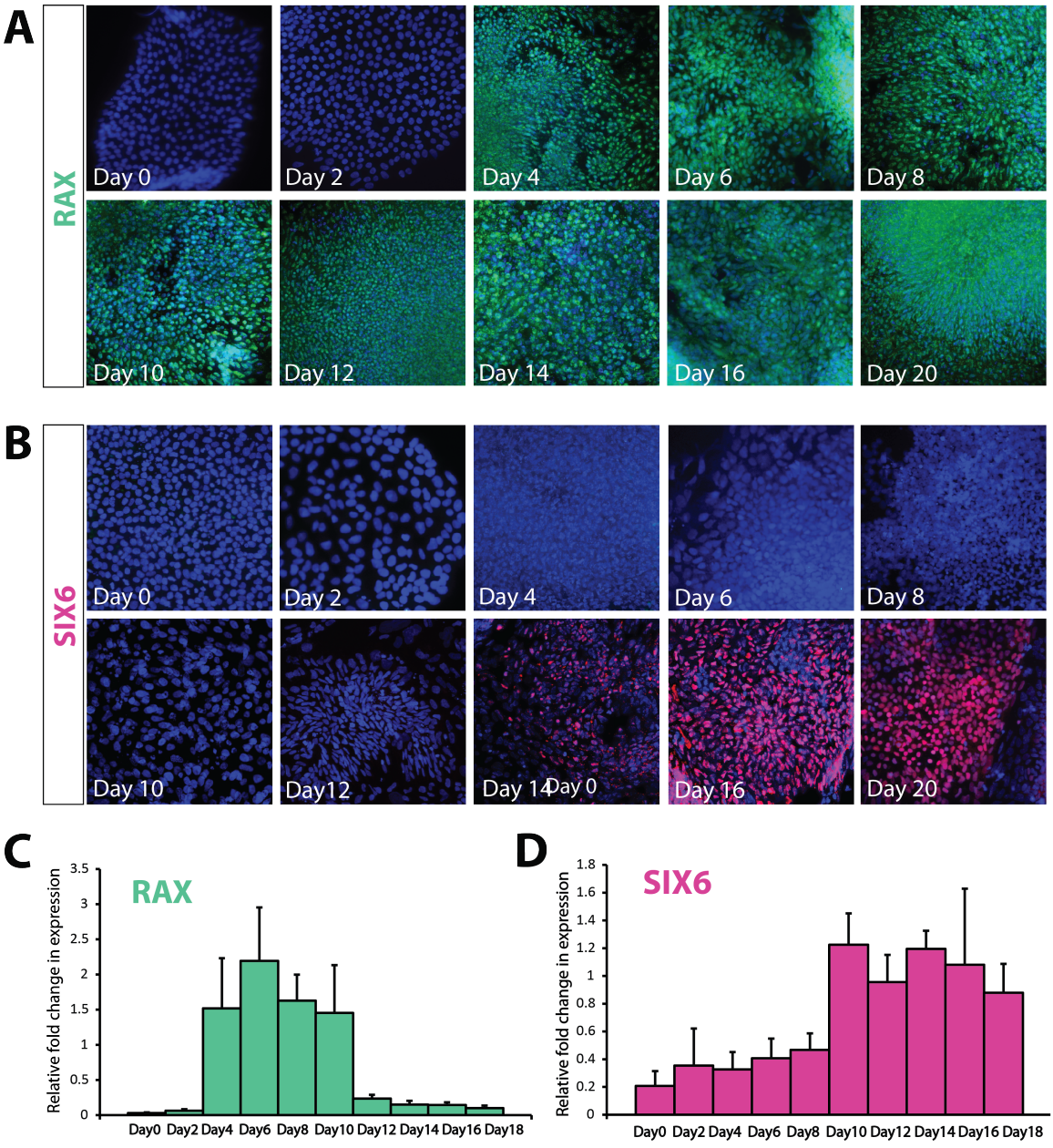

Figure 2.6. The expression of the eye field transcription factors *RAX* and *SIX6* varied in their expression patterns compared to the other EFTFs. ICC demonstrated the expression patterns of RAX, which began its expression around day 4 of differentiation (A) and SIX6 (B), which began its expression late, around day 14 of differentiation. *RAX* peaked early followed by a gradual reduction, presumably becoming restricted to a subset of retinal cells (C). The expression of *SIX6* was not observed until after the expression of other EFTFs (D), with an expression pattern found only within a subset of neurospheres by 20 days of differentiation.

Previous studies in the Meyer lab have indicated that *RAX* and *SIX6* are two transcription factors whose expression is restricted to the retinal cells at twenty of differentiation and absent in non-retinal cells at this time point [117]. However, the dynamic expression patterns of these transcription factors over time remained to be further characterized. Immunocytochemistry results from our work indicated an early onset of RAX expression beginning at day 4 of differentiation while SIX6 has a late, delayed onset of expression, starting at day 14 of expression (Figure 2.6A,B). Interestingly, Q-PCR indicates variable trends in the expression of these factors. *RAX* expression increased until day 10 of differentiation followed by a sudden decrease in expression at day 12 of differentiation (Figure 2.6C). The level of expression of *RAX* at day 12 is still higher than the expression levels at Day 0, indicating decrease in expression as opposed to loss of *RAX* expression, potentially indicating that RAX expression is retained in those cells committed to become retinal cells. After day 12, the expression of *RAX* is retained at a constant level for twenty days of expression. Hence, the trends for *RAX* based on the QPCR data seem to indicate the retention of *RAX* expression exclusively in the cells which will adopt a retinal phenotype after day 12 of expression.

Interestingly, *SIX6* peaks around day 12 of differentiation, about the time that *RAX* expression levels drop (Figure 2.6D). Also, *SIX6* expression is appeared to be exclusive to the retinal cells by twenty days of differentiation. This data allows for the possibility that the maintained expression of *RAX* 'primes' the cells to adopt a retinal phenotype, with the subsequent expression of *SIX6* committing these cells to a retinal fate. Hence, this data indicates the applicability of *RAX* and *SIX6* as candidate transcription factors playing significant roles in specifying a retinal fate from an unspecified stem cell source.

2.4 Discussion

The specification of the retina is a complex process, regulated by a variety of bHLH factors, transcription factors and homeobox genes such as EFTFs [11, 12, 97]. Previously, animal models such as *Xenopus* and mouse have been used to study and model human retinogenesis [8,140]. However, the evolutionary differences between lower vertebrate models such as *Xenopus* and humans slightly limit the applicability of such animal-based studies to effectively model human development [98, 99]. The advent of Human pluripotent stem cells (hPSCs), including both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), help overcome some of the disadvantages associated with traditional model systems such as *Xenopus* or mouse and hence, serve as powerful *in vitro* models for human developmental studies and genetic diseases.

A variety of genes, including EFTFs, have been known to play an important role in the development of retina in lower vertebrates [14]. The roles of EFTFs have been well characterized in *Xenopus* and mouse [8, 12] but their patterns of expression in higher organisms, including humans, have been limited. The data presented here is the first demonstration of the expression patterns of EFTFs in humans with the use of hiPSCs as an *in vitro* model. This study highlights the highly dynamic and overlapping patterns of expression of EFTFs in cultures of hiPSCs over twenty days of development. EFTFs like *LHX2, TBX3, SIX3, NR2E1* and *PAX6* have similar patterns of expression: they begin their expression around day 4 of differentiation and are retained in hiPSC cultures till twenty days of differentiation (Figure 2.5). However, two EFTFs, *RAX* and *SIX6* demonstrate differences in their expression patterns which indicate a more prominent role of these factors in specifying a retinal fate (Figure 2.6). Based on this data, *RAX* and *SIX6* were identified as candidate

transcription factors involved in specifying a retinal fate from a primitive anterior neuroectoderm population. Future studies involving gene overexpression or knock-down of these candidate transcription factors will help discern the exact roles of these factors in specification of a retinal fate.

3. DERIVATION OF RETINAL CELLS FROM hiPSCs UNDER XENO-FREE CONDITIONS

3.1 Introduction

Human induced pluripotent stem cells (hiPSCs) have been implicated to hold great potential for regenerative medicine through the unique ability to generate patient specific cell lines, which account for fundamental differences within the cells of individual patients and may potentially promote the development of a personalized treatment profile for a wide spectrum of diseases [100, 101]. However, significant obstacles still remain before this potential is realized due to the possibility of graft rejection and zoonosis because of the use of animal products or other undefined components during routine culture of these cells which needs to be addressed before effective cell replacement therapy can be warranted [84, 102]. Hence, a major goal of this field of research is the development of xeno-free differentiated progeny derived from hiPSCs which could then be successfully used for translational research and regenerative medicine.

Traditionally, human pluripotent stem cell (hPSCs) including human embryonic stem cells(hESCs) and hiPSCs, have been grown on a layer of mitotically inactivated Mouse Embryonic Fibroblasts (MEFs) in the presence of medium containing Fetal Bovine Serum (FBS) or Knock Out-Serum Replacement (KOSR) allowing for the presence of animal components under these growth conditions [86, 92, 103–106]. More recent efforts have been made to eliminate the use of undefined growth conditions through the use of chemically defined media [107–110]. However, such approaches often rely upon defined animal proteins in the medium, as well as the growth of cells upon a matrigel substrate [111]. More recent efforts have been focused on the growth of

hPSCs under xeno-free conditions, along with the differentiation to limited cellular lineages [112–115]. However, the successful growth and differentiation of hPSCs to a retinal lineage has been largely unexplored. Thus, for translational purposes, a need exists to enable the growth and differentiation of hPSCs in the absence of xenogeneic materials.

We have previously demonstrated the derivation of a variety of retinal cells from hPSCs including photoreceptors, retinal ganglion cells and RPE using a stepwise differentiation protocol [93, 116, 117]. In the current study, we have adapted this procedure to allow for the successful growth and differentiation of hiPSCs to a retinal phenotype in the absence of xenogeneic materials. Care was taken to ensure all ancillary components used in the process of differentiating hiPSCs were free of animal origin to reinforce the applicability of hiPSCs to the field of translational medicine. Here, we present a xeno-free technique for growth and differentiation of hiPSCs into retinal cells that closely resembles previously established methods for derivation of retinal cells, but is devoid of xenogeneic factors.

3.2 Methods

3.2.1 Maintenance of undifferentiated colonies

IMR90-4 hiPSCs (WiCell) were maintained in the undifferentiated state under three different conditions; MEF, feeder-free (FF) and xeno-free (XF). The MEF system required the growth of hiPSCs on MEF, with the supplementation of iPS medium (20% Knockout Serum, 0.1mM β-mercaptoethanol, 1mM L-glutamine, DMEM-F12(1:1), MEM Non-Essential Amino Acids, 4ng/ml FGF2) on a daily basis. The feeder-free system comprised of the growth of hiPSCs on matrigel and the use of mTESR1 medium (Stemcell Technologies) for maintenance of cells in an undifferentiated state.

Lastly, the xeno-free system consisted of cells grown on Synthemax plates (Corning) with the addition of Nutristem medium (Stemgent). The use of MEF and feeder-free system for the growth of hiPSCs has been reviewed extensively in literature [91–93, 111, 118, 119], allowing for their role as controls for our experiments.

Cells were passaged approximately every four days, usually when the colonies cells were 70-80% confluent. Colonies containing clearly visible differentiated cells were manually removed before passaging. The remaining colonies were lifted off the plate enzymatically by treatment with dispase (2mg/ml) followed by three washes with DMEM-F12. Colonies were then broken up into smaller clusters by manual trituration and were plated at a ratio of 1:6.

3.2.2 Differentiation of hiPSCs

Colonies of undifferentiated hiPSCs were directed to differentiate via the formation of embryoid bodies (EBs) through treatment with dispase, as described previously [93]. Cells grown on the MEF system were immediately transferred to Neural Induction Medium (NIM; DMEM-F12 (1:1), 1%N2 supplement, MEM Non-Essential Amino Acids, 2μg/ml Heparin) whereas cells grown under feeder-free and xeno-free conditions were slowly transitioned into NIM by transferring the EBs to a 3:1 ratio of mTESR1/Nutristem: NIM on day 0, 1:1 on day 1, 1:3 on day 2 followed by transfer to complete NIM at day 3 of differentiation.

At day 7 of differentiation, the cells grown on MEF and feeder-free system were plated on 6 well plates coated with laminin (20μg/ml). To maintain a non-xenogeneic environment for xeno-free cultures, laminin coating was omitted and Synthemax plates were used. Cells acquired advancing morphological features particular to neural rosettes over 17 days of differentiation, followed by their transfer to Retinal Differentiation Medium (RDM; DMEM-F12 (3:1), 2% B27, non-xenogenic B27 was used

for XF system). Retinal neurospheres identified by a bright ring appearance around the periphery were manually picked as previously established (Meyer et al., 2009). Neurospheres were fed every 3-4 days and were maintained in suspension upto 60 days of differentiation. For RPE differentiation, cells were kept adherent at day 17 of differentiation and were fed with RDM every 3-4 days until approximately 60 days of differentiation.

3.2.3 Immunocytochemisty

Samples were collected at specified timepoints of differentiation and were plated onto coverslips coated with poly-ornithine/laminin (20μg/ml), fixed with 4% paraformaldehyde for 30 minutes followed by three washes with PBS for five minutes each. The cells were permeabilized by treating them with 0.2% trypsin for ten minutes followed by blocking it for an hour with 10% donkey serum. Primary antibody was added at the recommended dilution in 0.1% triton-X and 5% donkey serum and incubated overnight at 4 °C. The next day, primary antibody was removed and cells were washed 3X with PBS, followed by blocking with 10% donkey serum for ten minutes. The secondary antibody was diluted along with DAPI in 0.1% triton-X and 5% donkey serum for an hour. Samples were then washed with PBS and mounted and applied on slides using mounting medium.

In order to perform ICC on RPE, pigmented patches of cells were micro-dissected manually and re-plated on Poly-orinithine/laminin coated coverslips. RPE cells were supplemented with RDM containing FGF2 (20ng/ml), EGF (20ng/ml) and Heparin (2μg/ml) for a week. Mitogens were then removed for two weeks and cells were stained as described above.

3.2.4 RT-PCR

Cells were collected at specified time points of differentiation and RNA was extracted using PicoPure RNA Isolation Kit (Applied Biosystems). cDNA was made using the iScript cDNA Synthesis Kit (Bio-Rad) or SuperScript III First-Strand Synthesis System (Invitrogen). cDNA was used for RT-PCR using GoTaq qPCR Master Mix (Promega). PCR was performed for 30 cycles followed by analysis of PCR products on 2% agarose gels.

3.3 Results

3.3.1 Examining features of pluripotency under different conditions

The ability to effectively expand hiPSCs and maintain pluripotency is essential for future applications. Thus, experiments were designed to analyze pluripotency characteristics in hiPSCs cultures under the three conditions. After a minimum of five passages in either MEF, feeder-free or xeno-free systems, colonies of hiPSCs exhibited a uniform appearance without marked differences in the morphologies of the colonies under the different culture conditions (Figure 3.1A). Under all three conditions, immunocytochemistry results confirmed the expression of key pluripotency associated factors in colonies of hiPSCs including OCT4, SOX2, NANOG, SSEA-4, TRA-1-60, and TRA-1-81 (Figure 3.1B-G). Maintenance of the pluripotent state was further confirmed through RT-PCR analysis, where key pluripotency genes were found to be expressed under all conditions (Figure 3.1H). In addition to the expression of characteristic pluripotency genes, colonies of hiPSCs grown under each culture condition also tested negative for the expression of differentiation markers including α-*FETOPROTEIN*, *PAX6* and *BRACHYURY*, further confirming their undifferentiated state.

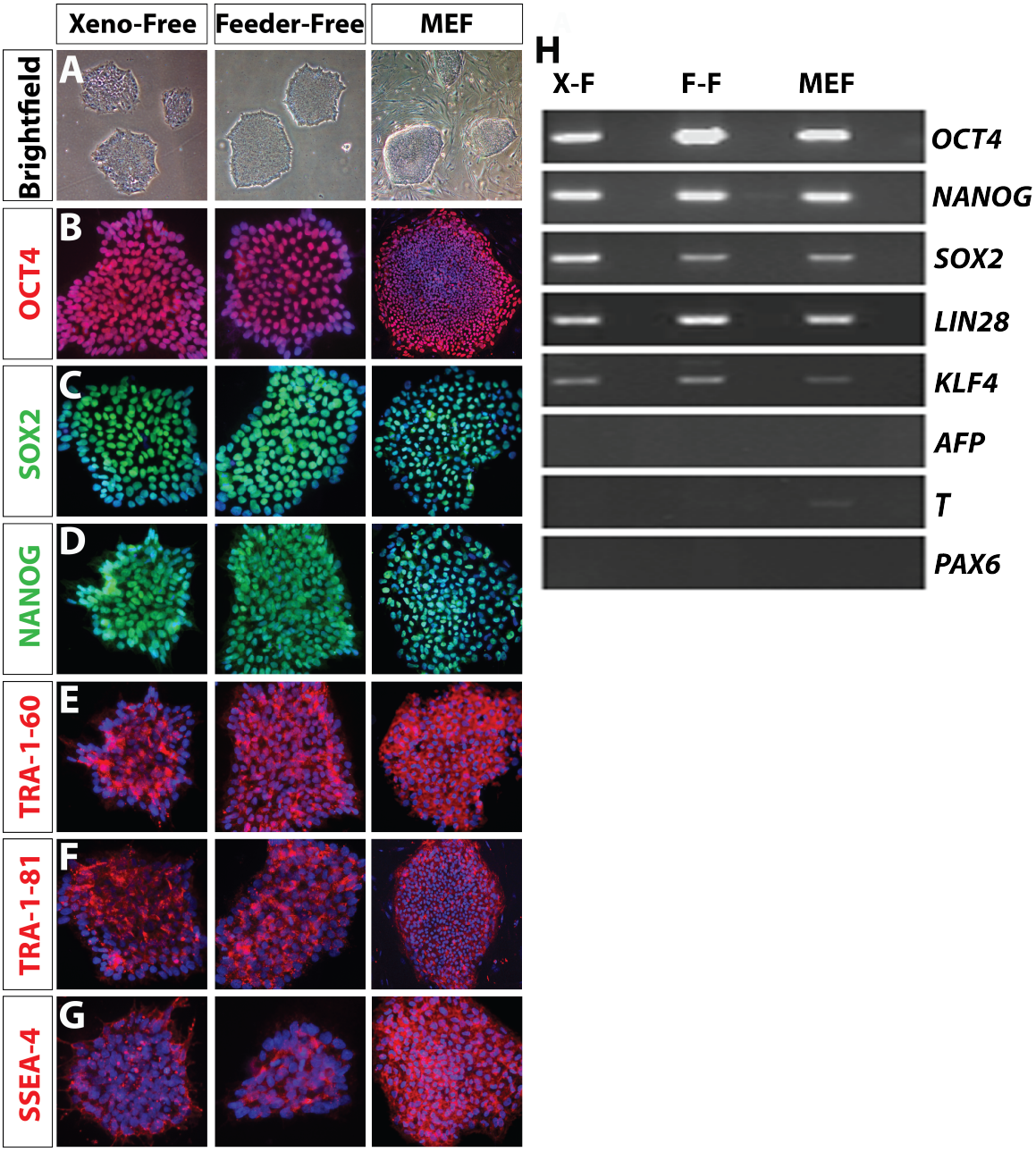

Figure 3.1. Colonies grown under xeno-free, feeder-free and MEF system of development exhibited similar morphological features when viewed under bright field microscope (A). Uniform and homogenous expression of pluripotency-associated factors like OCT-4, NANOG and SOX2 was observed in the undifferentiated colonies, irrespective of the system they were grown in (B-D). Similar expression of cell surface markers like SSEA-4, TRA-1-61 and TRA-1-81 in the xeno-free cells when compared to traditional systems further confirm the pluripotency characteristics of these cells (E-G). RT-PCR recapitulates the trends observed in ICC and confirmed the presence of the pluripotency-associated transcription factors such *OCT4, NANOG, SOX2, LIN28* and *KLF4* and the absence of factors associated with differentiation like *PAX6* (H).

3.3.2 Specification of neural and retinal progenitor cells

Prior to the specification of mature retinal cells types, hiPSCs must be prompted to differentiate through the major stages of retinal development including those stages analogous to primitive eye field, optic vesicle and optic cup stages. Following modifications to previously established protocols, hiPS cells initially acquired features of primitive anterior neuroepithelium, including the eye field. Immunocytochemistry and RT-PCR experiments illustrated the expression of transcription factors *SOX1*, *SOX2*, *PAX6* and *OTX2* (Figure 3.2A-D), which were indicative of the acquisition of an anterior neural phenotype. Importantly, inappropriate regional and temporal gene expression was generally not observed within these cells, confirmed by the lack of expression of markers associated with other germ layers and posterior markers of the midbrain and hindbrain (*HOXB4, ENI*; Figure 3.2G). The establishment of anterior neural identity was also indicated by the emergence of the eye field stage, characterized by the expression of EFTFs [12] *PAX6*, *RAX*, *SIX3*, *SIX6*, *LHX2* and *NR2E1* (Figure 3.2G). Immunocytochemistry and RT-PCR experiments demonstrated coordinated and overlapping expression of EFTFs in differentiating iPS cell colonies. Moreover, the expression patterns of all transcription factors at this stage were similar under all three conditions tested, illustrating the potential to derive retinal cell types under non-xenogeneic conditions (Figure 3.2G).

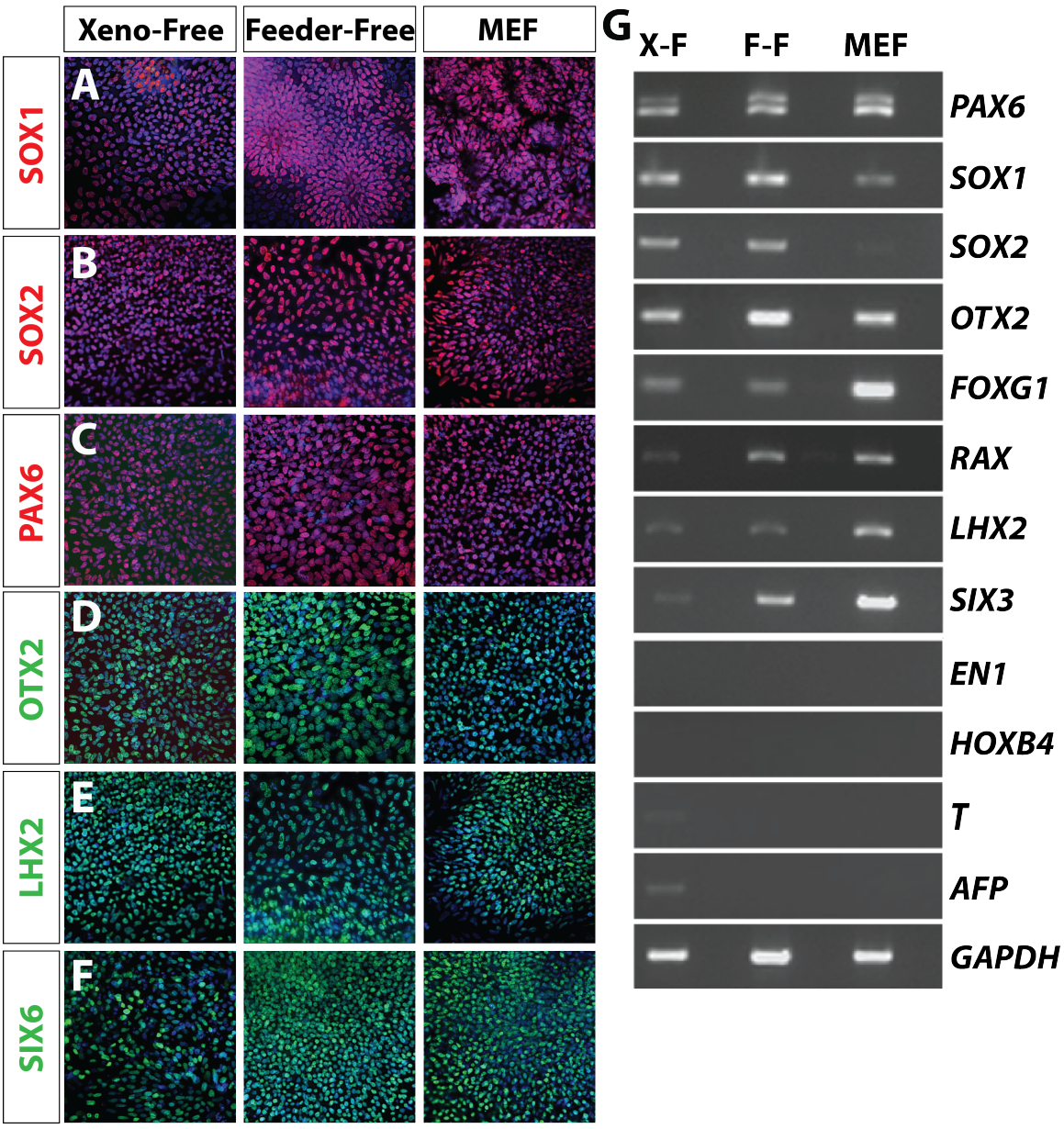

Figure 3.2. Following modifications of established neuroretinal induction protocols, hiPS cells acquired features of the primitive anterior neuroepithelium, including the eye field. Expression of SOX1, SOX2, PAX6 and OTX2 confers a broad neural identity to these cells (A-D). Expressions of numerous genes/proteins associated with the eye field stage such as LHX2 and SIX6 were observed within these cells after10 days of differentiation (E, F). RT-PCR highlights the expression of all the transcription factors associated at this stage of development. Importantly, inappropriate regional and temporal gene expression was generally not observed within these cells (G).

Following the acquisition of a primitive eye-field like phenotype, hiPSCs must be directed to differentiate towards subsequent stages of retinal development, including those analogous to the optic vesicle and optic cup. We have previously demonstrated the ability to identify and isolate two morphologically distinct populations of cells in cultures of differentiating hiPSCs within the first twenty days of differentiation with characteristics analogous to retinal and forebrain progenitor cells [93, 117]. Neurospheres possessing a bright ring around the periphery were isolated via bright field microscopy and expressed a full complement of retinal progenitor cell-associated transcription factors such as *CHX10* (Figure 3.3A,B), *MITF* and *TBX2* (Figure 3.3F). Immunocytochemistry and RT-PCR experiments demonstrated the expression of these transcription factors in xeno-free, feeder free and MEF conditions (Figure 3.3A-F). The expression of some anterior retinal transcriptions factors such as *PAX6*, *LHX2*, *SIX6* (Figure 3.3C-E) and *RAX* (Figure 3.3F) were also maintained in these spheres. The absence of mature retinal markers such as *CRX* and *BRN3*, along with expression of *CHX10* was suggestive of a retinal progenitor cell population (Figure 3.3F). The consistency of expression of these transcription factors across all three systems further illustrates the applicability of this differentiation protocol to different systems (Figure 3.3F).

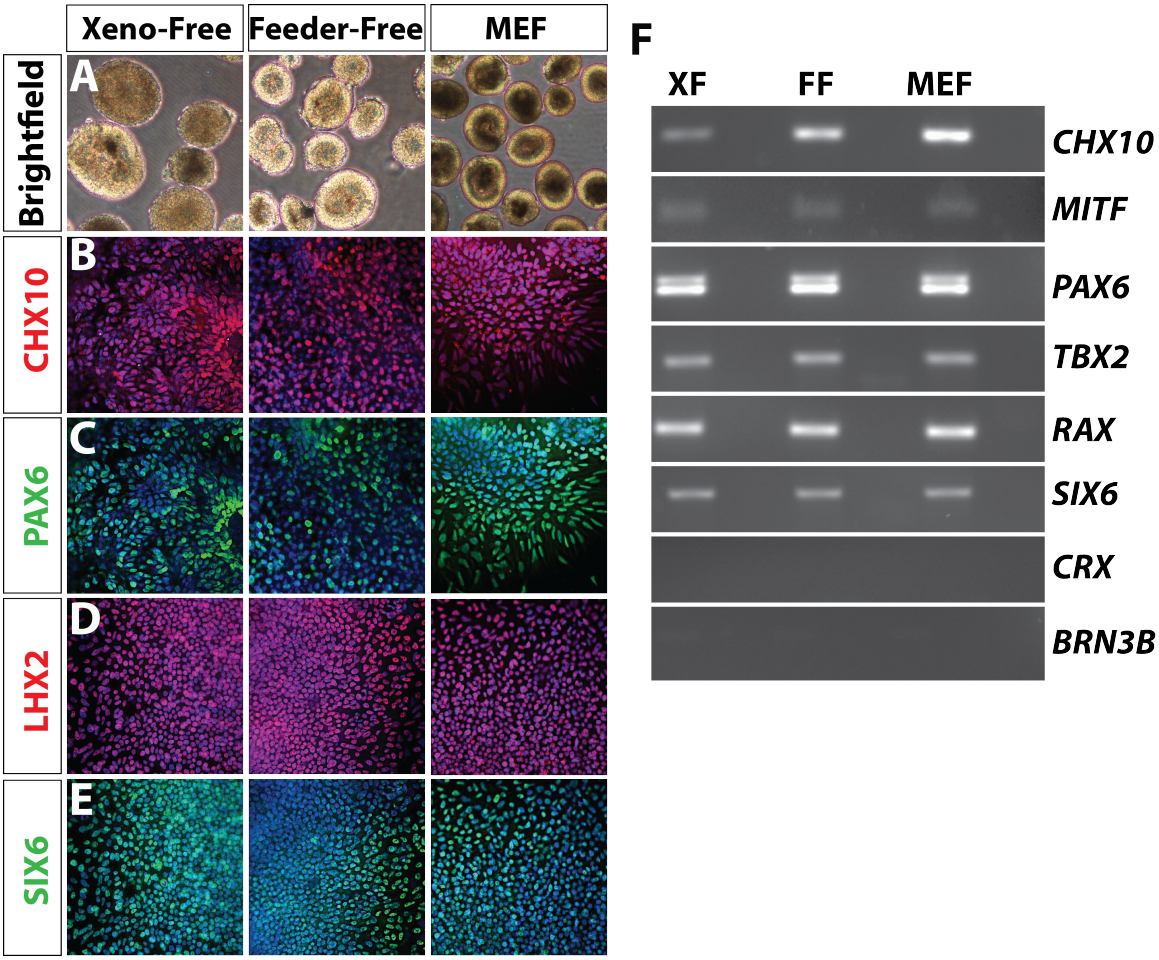

Figure 3.3. Under varying growth conditions, retinal progenitor spheres can be isolated via brightfield microscopy. The retinal spheres were identified by a light outer ring around the periphery, a morphological feature absent in non-retinal spheres (A). The retinal cells stained positive for CHX10, a marker for retinal progenitor cells (B). The cells grown on all three systems retained the expression of EFTFs like PAX6, LHX2 and SIX6 (C). RT-PCR data confirms ICC trends and indicates similar expression levels of cells grown in a non-xenogenic environment when compared to the cells grown using traditional systems. More mature markers like *CRX* and *BRN3* are absent at this stage (D).

As opposed to those neurospheres that gave rise to retinal progenitor cells, the other neurospheres were previously demonstrated to be composed of forebrain progenitor cells. These neurospheres contained neural progenitors of an anterior iden-

tity (*SOX1*/*PAX6*/*OTX2*-positive) as well as βIII-TUBULIN positive neurons (Figure 3.4A-D). As demonstrated by RT-PCR analysis, these neurospheres expressed a full set of anterior neural transcription factors such as *FOXG1*, *DLX1* and *GSX2* (Figure 3.4E). These results demonstrates that the ability to enrich for retinal progenitor cells apart from other neural cell types is maintained under xeno-free conditions, as previously established for traditional systems.

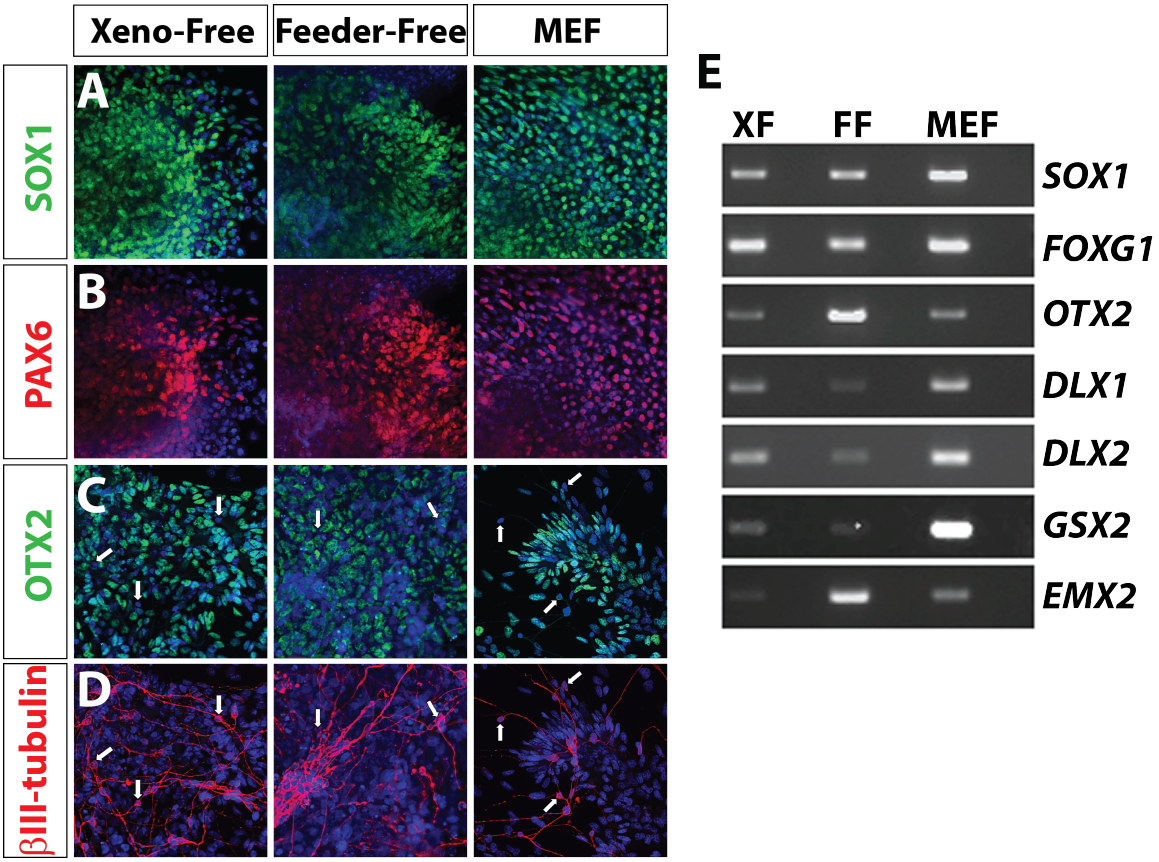

Figure 3.4. Non-retinal neurospheres express markers indicative of anterior neural phenotype like PAX6, OTX2 and SOX1 at 25 days of differentiation (A-C). The loss of OTX2 expression in some cells is confirmed by the presence of more mature neuronal markers like βIII-tubulin neurons, indicated by white arrows (C,D). RT-PCR analysis demonstrates that these cells express a full complement of anterior neural transcription factors (E).

3.3.3 Differentiation of mature retinal cell types

For future translational applications, it will be necessary to derive more retinal cells under non-xenogeneic conditions, including cells of both RPE and neural retina. In the current study, hiPSCs could be directed to become RPE under xeno-free conditions, similar to previous documentation in the MEF system. Pigmented, hexagonal RPE-like cells were first apparent approximately one month following the start of differentiation and increased over next few weeks (Figure 3.5A). Immunocytochemistry at 50 days of differentiation revealed the expression of RPE-associated tight junction proteins such as ZO-1 as well as RPE associated transcription factor OTX2 (Figure 3.5B). RPE from both xeno-free and traditional cultures expressed a full complement of RPE-associated genes such as *RPE65*, *PEDF*, *ZO-1*, *MITF* and *BEST1* which were confirmed by RT-PCR (Figure 3.5C).

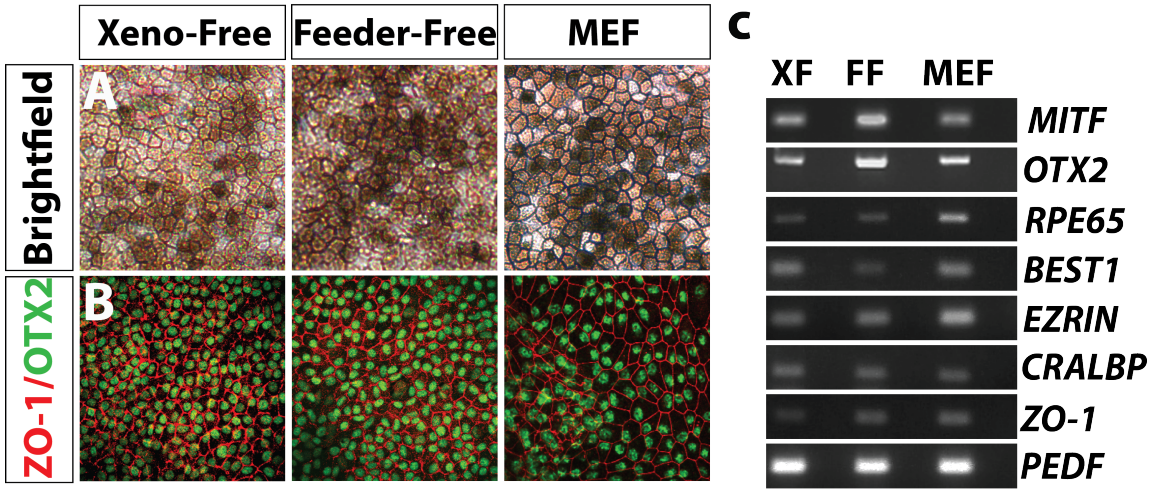

Figure 3.5. Bright field images confirm the presence of pigmented, hexagonal RPE-like cells. These were first apparent approximately one month following the start of differentiation and increased over the next few weeks (A). RPE retained the expression of OTX2 and stained positive for ZO-1, a tight-junction protein associated primarily with epithelial cells (B). RT-PCR from xeno-free and traditional cultures expressed a full complement of RPE-associated genes (C).

Beyond the ability to derive RPE cells, it will also likely be necessary to derive cells of the neural retina under non-xenogenic conditions, including photoreceptors and retinal ganglion cells. In the current study, we observed cells that had acquired morphologies of primitive photoreceptor-like cells *in vitro*, after 50 days of differentiation (Figure 3.6A). Cells expressed genes associated with varied neural retinal phenotypes, with numerous cells expressing the photoreceptor precursor-specific transcription factor CRX as well as RECOVERIN, indicative of a cone-photoreceptor fate (Figure 3.6B). Additionally, other cells expressed BRN3, indicating the existence of retinal ganglion cells in those cultures (Figure 3.6C). RT-PCR demonstrates the acquisition of advancing features of retinal cells over 50 days of differentiation in a xeno-free environment while mirroring the developmental process as previously documented in traditional systems.

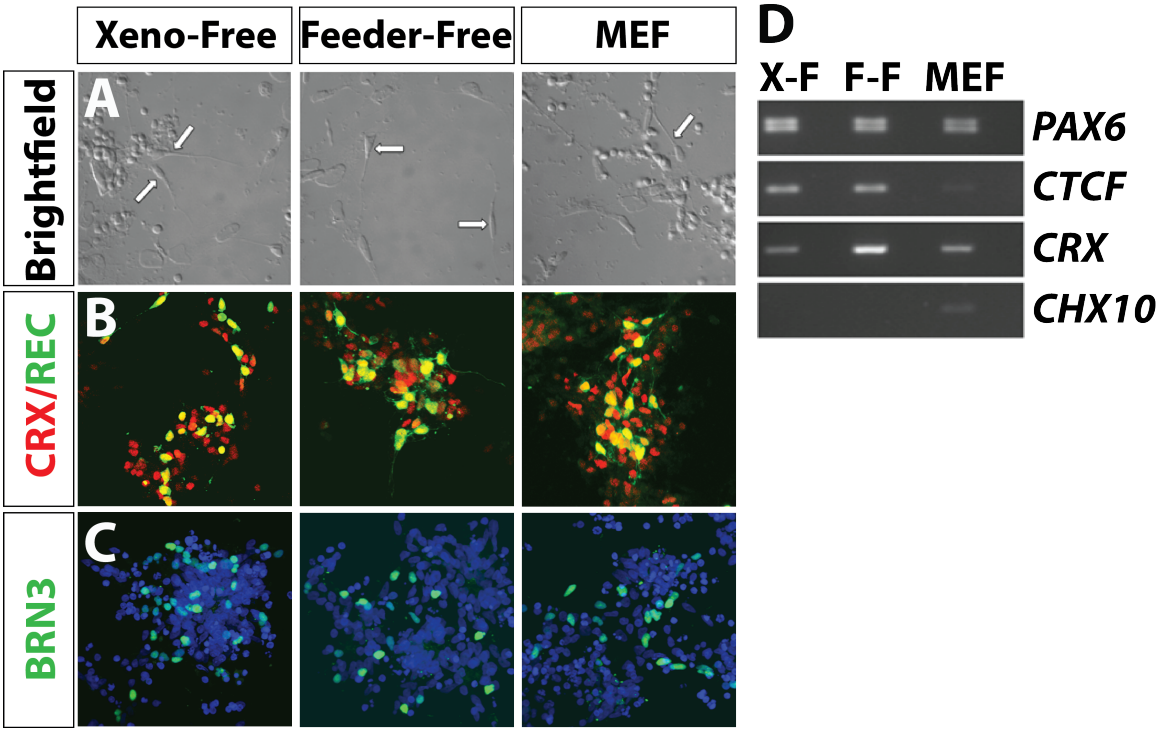

Figure 3.6. After 50 days of differentiation, cells acquired morphologies of primitive photoreceptor-like cells *in vitro*, including a unipolar appearance with one short process, as indicated by the arrows (A). Cells expressed genes associated with varied neural retinal phenotypes, with numerous cells expressing the photoreceptor precursor-specific transcription factor *CRX* and *RECOVERIN* (B). Cells also express markers such as *BRN3*, which are specific to retinal ganglion cells (C). RT-PCR indicates the expression of genes specific to different retinal subtypes such as *CRX*, which is specific to photoreceptors (D). Due to acquisition of mature markers, the retinal progenitor marker *CHX10* is no longer expressed.

3.4 Discussion

The data presented here demonstrates the ability to derive retinal cell types from hiPSCs via a differentiation protocol that excludes the presence of xenogeneic components. We establish the feasibility to generate retinal cell types, including RPE,

photoreceptors and retinal ganglion cells, from hiPSCs in a xeno-free manner with minimal variability as compared to those cells derived by traditional methods of differentiation. Such ability will likely be of great importance as hiPSC research is developed for more translational purposes.

Several reports have focused upon the ability to derive retinal cell types from hiPSCs. However, most of these studies have relied upon xenogeneic culture systems utilizing media containing animal products and/or mouse feeder cells to support the growth of hiPSCs [104, 120–123]. The ability to derive retinal cells from hiPSCs under non-xenogeneic conditions could have potentially profound implications for future approaches to the treatment of retinal degenerative disorders, including age-related macular degeneration and glaucoma. More recently, a few reports have focused on the ability to derive RPE cells in a less xenogeneic culture environment [85, 119, 121, 124–126]. Such studies have successfully generated RPE cells, but these systems have remained somewhat xenogeneic, in that, the use of FBS in the medium raises possible cases of zoonosis [124]. Furthermore, studies to date have excluded the ability to derive cells of the neural retinal including photoreceptors and retinal ganglion cells [127].

To build upon previous studies and establish a truly non-xenogeneic system with which to derive retinal cells from hiPSCs, we sought to maintain a xeno-free environment in our system in a two fold manner: Firstly, a defined, feeder-free alternative for the growth of hiPSCs was developed through the use of synthetically-coated culture plates. Secondly, media devoid of xenogeneic components was used to ensure the maintenance of a xeno-free environment through the course of the experiments, including both expansion of hiPSCs as well as their differentiation to a more mature retinal lineage. The ability to derive retinal phenotypes under non-xenogeneic conditions

also has important implications for the development of stem cell-based approaches to a variety of disorders, as many groups have demonstrated the ability to derive a variety of neural cell types following similar differentiation paradigms [92, 128].

The results presented in this study offer numerous advantages over previously described approaches to derive retinal cells from hiPSCs [92, 106]. First, the non-xenogeneic culture conditions established within this manuscript are completely chemically defined, whereas previous studies often relied upon the use of serum or knockout serum-containing medium at some stage of the differentiation process [124, 129]. Additionally, beyond the use of commercially available media supplements, this method does not require the use of additional exogenous growth factors which may complicate efforts to establish a non-xenogeneic culture system. Furthermore, as previously demonstrated under traditional culture systems [117], we establish the ability to identify and enrich for retinal progenitor cell populations based upon morphological characteristics, and now demonstrate this capability under non-xenogeneic conditions. Such an ability to derive essentially pure populations of retinal progenitor cells under non-xenogeneic culture conditions will likely be essential as translational applications for hiPSCs are developed.

The results presented in this study establish the proof-of-principle that hiPSCs can be specified to differentiate into mature neural cell types such as retinal neurons under non-xenogeneic conditions. Such results are likely to have important implications as new stem cell-based approaches are developed for the treatment of human disease. This is of particular importance as the first clinical trials for human embryonic stem cell-based products are underway for the potential treatment of age-related macular degeneration [130]. Before cells grown under non-xenogeneic conditions can be utilized in therapeutic applications, other precautions will likely be necessary. First, if existing cell lines are utilized, it may be necessary to demonstrate that xenogeneic material has not been retained by these hiPSCs. Alternatively, it may be advanta-

geous to derive new lines of hiPSCS under non-xenogeneic conditions, as described previously for hESCs [131]. Additionally, routine culturing of these cells in a research lab does not afford the same level of protection for a patient as those cells grown under Good Manufacturing Practices (GMP). Before the translation of this research to therapeutic applications, it will likely be advantageous to expand the results presented here to include the differentiation of these cells under GMP-compliant environmental conditions.

In summary, the results of this manuscript demonstrate the ability to differentiate hiPSCs into a variety of retinal cell types under non-xenogeneic culture conditions. This study represents the first demonstration of non-xenogeneic differentiation of hiPSCs into neural retinal cell types such as photoreceptors and retinal ganglion cells, which is likely to have important implications for the treatment of diseases such as age-related macular degeneration and glaucoma. Of importance, no marked differences in the maintenance and differentiation of hiPSCs into retinal cells were observed between each of the three culture conditions. The results of this study also highlight the applicability of non-xenogeneic growth and differentiation of hiPSCs to other systems for translational applications. While additional studies are still necessary before widespread application of hiPSCs for translational applications, the current study serves to establish the feasibility of non-xenogeneic growth and differentiation of hiPSCs for applications related to retinal development and disease.

4. DISCUSSION

Human induced pluripotent stem cells (hiPSCs) are capable of differentiating into any cell type of the body. Hence, they possess the ability to be directed to generate a specific subset of cells, including nerve cells, muscle cells or blood cells. Due to this ability, hiPSCs have been demonstrated to serve as an important tool for studying human development, particularly, the development of neural structures such as the retina. The ease of accessibility of the retina, as well as its highly organized structure and a thorough understanding of the neural circuits supports the use of retina as the popular system for hiPSCs mediated cell replacement.

Some of the earliest experiments to derive retinal cells from human stem cell populations were performed utilizing hESCs [86, 91, 93, 119, 132]. In order to model retinogenesis *in vitro*, one of the first steps is to direct hPSCs to an anterior phenotype. This has traditionally been accomplished through the use of inhibitors to block BMP and WNT pathways to direct the cells to an anterior-neural fate [86]. However, these compounds are expensive and often require cellular exposure during precise windows of development. Additionally, it is necessary to ensure that *in vitro* development of retinal cell types mirrors what is known about retinogenesis *in vivo*. For example, in humans, rod photoreceptors are among the last cell types of the retina to develop, as they are generated within first 100-120 days of development. However, some differentiation protocols demonstrate the appearance of photoreceptor markers within the first 30 days of differentiation [86]. Hence, although a variety of protocols are efficient, they fail to successfully recapitulate retinal development.

Hence, efforts have been made to devise a differentiation protocol that mirrors human retinogenesis. Based on these, a group in Japan derived retinal cells from hESCs us-

ing a different set of inhibitors [91]. Initially, they used small molecule inhibitors to direct the cells to an anterior neural fate. Later, the cells were induced to form photoreceptors using retinoic acid and taurine. However, these cells arose within highly mixed populations of cells and required culturing periods of up to 180 days to achieve a photoreceptor fate.

Thus, a need existed to develop a protocol that allowed for the prospective isolation of retinal progenitor cells and more closely mirrored the events known to occur during normal human retinogenesis. Hence, the Meyer lab developed a protocol to successfully direct pluripotent stem cells to a retinal fate, which takes in to account the limitations of earlier protocols while presenting an efficient method to derive retinal cells. One of the important issues emphasized by this protocol is the importance of choosing the right cell line. It was established that different cell lines respond differently to different growth factors and vary in their capability to adopt a neural fate [93]. For example, some cell lines block BMP and WNT signaling on their own while others did not demonstrate this ability [117]. Hence, based on this study, we initially chose the hiPSC line IMR90-4 for our developmental studies.

The cell line IMR90-4 has been used by several labs previously to generate neurons in a culture dish [21, 117, 133]. Since neurogenesis occurs before retinal specification, these cells possess the capability to be directed towards a retinal fate. One of the unique advantages of this cell line is that it blocks BMP and WNT signaling without the addition of external inhibitors like Noggin and Dkk-1 [117]. Also, it has been demonstrated that these cells adopt an anterior neural phenotype as their default neural phenotype. Hence, upon spontaneous differentiation, these cells express *PAX6*, a broad neural marker, as well as many other markers of an anterior neural fate including *OTX2* and *BF-1*, hence acquiring an anterior-neural phenotype [21].

The cells were directed to become cells of the retina in a manner that recapitulates normal human retinogenesis [93, 117]. In order to study development *in vitro*, transcription factors unique to each developmental stage were analyzed (Figure 1.4). Hence, using this approach, cells at each stage of differentiation could be identified on the basis of the markers expressed. Not only does this protocol faithfully document retinogenesis, it does so without the use of any exogenous growth factors or inhibitors. At each stage of development, the colonies exhibit morphological features reminiscent of stages of human development (Figure 4.1). For example, neural rosettes observed in cultures from Day 12-Day 16 of differentiation were reminiscent of closure of the neural tube during neurogenesis *in vivo* [105, 135].

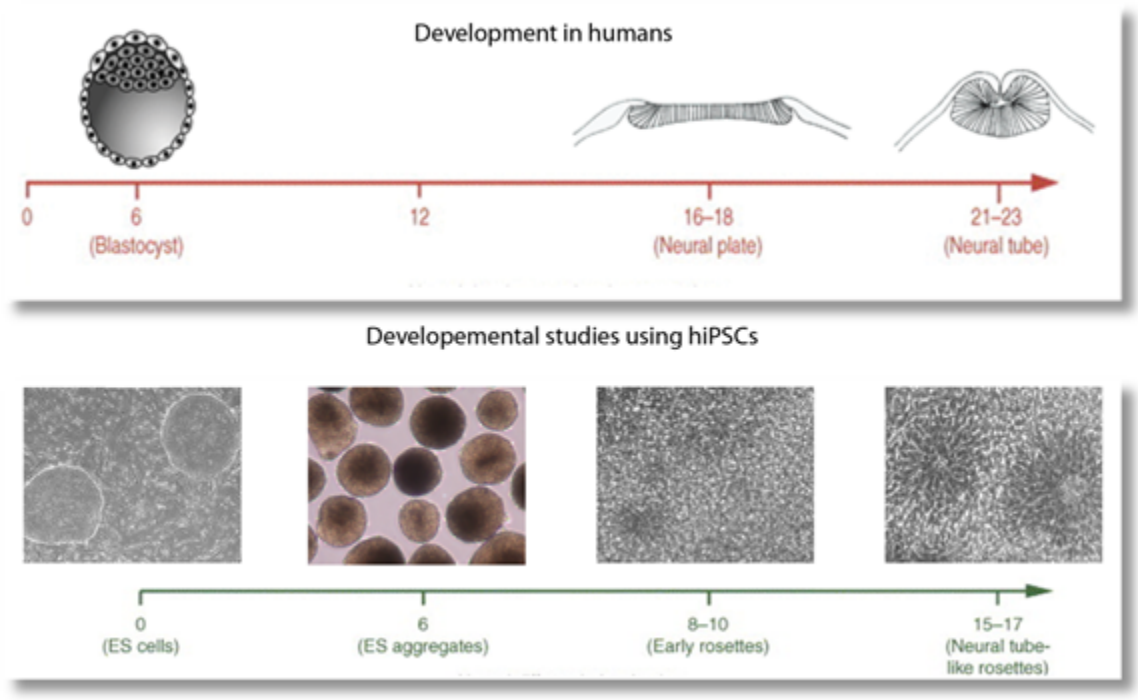

Figure 4.1. Neurogenesis in humans (top) is recapitulated by hiPSCs *in vitro* (bottom). Image modified from [93, 138].

Previous studies have emphasized the use of growth factors to drive cells to a retinal fate [86, 91]. In order to identify retinal cells in cultures, the cells are screened

for transcription factors such as *CHX10*, which is an indicator of retinal progenitor cells and later factors including *BRN-3* and *CRX*, specific to retinal ganglion cells and photoreceptors, respectively [139]. However, most techniques used to evaluate the presence of transcription factors require that the cells have been already fixed or lysed. Hence, an efficient way of identifying retinal progenitors in live cultures was developed in the Meyer lab.

After twenty days of differentiation, cells were transferred to a suspension culture where they rounded up and organized themselves into three-dimensional spheres [93, 117]. Upon observation under a bright field microscope, two distinct populations of cells with different morphological features could be seen. Some spheres exhibited a light donut-shaped ring on the periphery while other spheres did not. Interestingly, the cells with the light ring around the periphery were reminiscent of the optic vesicle stage of human development in both their organization as well as their pattern of gene expression. The ability of these spheres to give rise to the optic cup structure which later develops into a stratified retina has also been recently demonstrated using mouse embryonic stem cells [136]. Similar studies involving blood derived human iPS cells (TiPS-5 cell line) have demonstrated positive staining for retinal ganglion cells and photoreceptors in these retinal spheres at day 80 of differentiation [137].

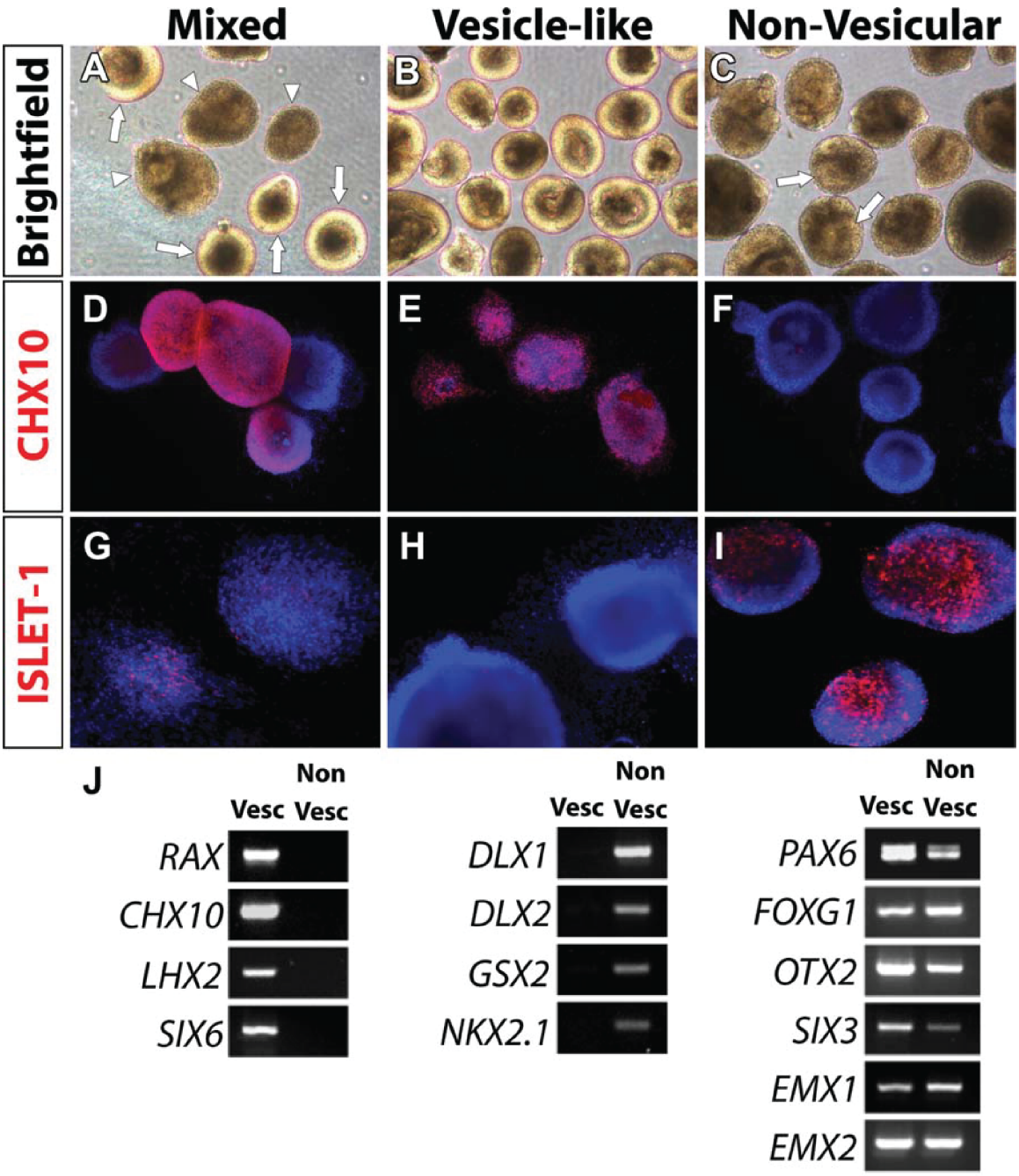

Figure 4.2. Two morphologically distinct populations arise from a mixed population of cells at day 20 of differentiation (A). Cells possessing a bright outer ring were classified as vesicular spheres and expressed markers specific to retinal progenitor cells such as CHX10 (B,E). Non-vesicular spheres lack the bright-outer ring and therefore lack the expression of CHX10 and are positive for ISLET-1 instead (C,F,I). Different set of transcription factors are expressed by vesicular and non-vesicular neurospheres (J). Image modified from [117].

Upon analyzing the populations further, the spheres not only exhibited morphological differences but also expressed a different set of transcription factors. Cells with unique morphological features expressed *CHX10*, a marker for retinal progenitor cells (Figure 4.2) They also expressed other transcription factors including *LHX2, RAX* and SIX6 which were unique to this subset of cells. Also, the spheres void of the light outer ring expressed anterior neural, yet non-retinal markers including *ISLET-1, SOX1* and *DLX5*. Hence, spheres with the light outer ring were categorized as retinal spheres while other spheres were called non-retinal spheres. However, the mechanisms underlying the emergence of these two populations have not been explored in detail. Hence, we decided to investigate the emergence of these populations in culture by studying retinal development using hiPSCs as a model system.

Retinal development is a complex interplay of a variety of signaling molecules such as BMP, FGF and WNT proteins, as well as the contributions of critical transcription factors [140]. Certain transcription factors have previously been demonstrated to play a more prominent role in retinal specification [141, 142]. Some of these factors have been very well characterized in lower organisms like *Xenopus* and are referred to as the EFTFs [12]. Interestingly, some of these EFTFs are initially expressed as early as 6 days of differentiation in hiPSC cultures and retained until 20 days of differentiation in the retinal spheres exclusively, a time point at which definitive retinal progenitor cell markers are expressed (Figure 4.2). Hence, we decided to further investigate expression patterns of EFTFs in hiPSC cultures.

The patterns of expression of EFTFs have been highly characterized in lower organisms such as *Xenopus* [12–14, 143]. However, their patterns of expression in retina and their role in specifying a retinal fate remain largely elusive in higher organisms, including humans. Hence, we decided to characterize their patterns of expression in hiPSCs to highlight their roles in retinal development.

hiPSCs were accessed for their loss of pluripotency-associated genes in a gradual manner to demonstrate the lack of any abnormalities in terms of differentiation of hiPSCs (Figure 2.2A-C). Next, hiPSCs were studied for the expression of genes indicative of establishment of the anterior neuroectodermal plate. Using animal models, it has been established that *Pax6* helps to determine the neuroectoderm in vertebrate development [21, 144, 145]. Also, *Pax6* plays an important role in eye development, particularly lens formation and specification of retinal cell types [146–148]. Hence, the expression of *PAX6* beginning in hiPSCs at day 6 of differentiation is indicative of the establishment of a primitive anterior neuroectodermal fate (Figure 2.3A,C). Also, it has been established that *Otx2* plays an essential role in the specification of the anterior neuroectoderm and is also retained throughout the optic vesicle stage and in the RPE of vertebrates [149, 150]. The expression of *OTX2* in our cultures highlights a similar trend in human development (Figure 2.3B,D). Interestingly, the expression of *OTX2* is observed as early as the undifferentiated state of hiPSCs, potentially elucidating a mechanism for the 'default' acquisition of an anterior neural phenotype within these cells.(Figure 2.3B).

Following the establishment of a neural fate, the next step of neurogenesis is the folding of the neural plate to form the neural tube. This is accompanied by the expression of definitive neural markers like *SOX1* and *DLX5* (Figure 2.4A-C). Experiments conducted in *Xenopus* show that *Sox1* expression directly correlates with the establishment of the neural ectoderm [151]. Experiments conducted in human embryonic kidney cells indicate similar functions of *SOX1* [152], which is further confirmed by our data (Figure 2.4A,C), indicating that *SOX1* promotes induction of neurogenesis in differentiating cultures of hiPSCs. The colonies form neural rosettes which express *SOX1* in a homogenous manner (Figure 2.4A).

The expression of EFTFs was investigated to determine their role in specifying a retinal phenotype in differentiating cultures of hiPSCs (Figure 2.5A-E). EFTFs have

been investigated in detail in lower organisms like *Drosophila* and *Xenopus* [12,14,15]. However, their role in the development of the human eye, specifically the retina, has not been characterized in detail. Studies of expression patterns of EFTFs indicates a uniform and spontaneous activation of transcription of genes including *PAX6, LHX2, NR2E1, TBX3* and *SIX3*, which begin their expression around Day 4 of differentiation. This is particularly interesting as experiments performed in *Xenopus* documented the expression of *nr2e1* in later stages of development [12]. However, our data indicates that *NR2E1* begins its expression earlier, at day 4 of differentiation, along with the onset of expression of many other EFTFs including *LHX2, SIX3, PAX6* and *TBX3* (Figure 2.5A-E). This difference between *Xenopus* and humans may underscore an important advantage of the use of hiPSCs over systems such as *Xenopus*. Given that the developmental process is considerably shorter in lower organisms, and that the expression patterns of these factors are highly similar and overlapping, it may not possible to discern the role of one factor in establishing retinal fate versus an anterior neural fate in this condensed timeline.

An interesting trend was seen in the expression patterns of other critical EFTFs including *RAX* and *SIX6* (Figure 2.6A-D). The function of *Rax* and its role in retinal development has been studied extensively in vertebrate systems such as mice [153, 155]. *Rax* is known to be important role in the formation of retinal progenitor cells and the regulation of eye development in mice [153, 154]. However, the role of *RAX* in human retinogenesis has not been studied extensively, often due to a lack of suitable experimental material. Our data is not only the first in vitro cellular model documenting the patterns and time course of *RAX* expression in humans but also highlights the role of *RAX* in specifying a retinal fate (Figure 2.6A,C). *RAX*

has an interesting pattern of expression where it begins its expression at day 4 of differentiation and increases its expression until day 10 of differentiation after which there is a sudden decrease, presumably becoming restricted to those cells that have been committed to a retinal progenitor cell fate (Figure 2.6C). This trend has been recently documented in the developing rat retina where densitometry quantification experiments indicate similar patterns of expression for *Rax* [156].

Since *RAX* is retained in the hiPSCs-derived retinal cells at twenty days of differentiation (Figure 3.3), the role of *RAX* in human retinal progenitor cells can be speculated upon. Experiments in *Xenopus* suggest that rax might play a role in controlling the proliferation of retinal progenitors as well as maintaining the progenitor cell population in a multipotent stage [157]. Studies have also shown *Rax* to be important in photoreceptor-cell fate specification at later stages of development [158]. Future studies will have to done to evaluate the role of *RAX* in human photoreceptor development. Nevertheless, based on previous literature and our data (Figure 2.6A,C), *RAX* stands out as an important candidate transcription factor in specifying a retinal fate.

SIX6 is another EFTF whose expression pattern makes it worthy of being considered as a candidate transcription factor for retinal development. The expression of *six6* has been well studied in *Xenopus* where overexpression of *six6* has been shown to lead to the formation of giant eyes with an accompanying expansion of retinal territory [24, 28]. The expression pattern of *SIX6* in hiPSCs was similar to what has been documented previously in lower vertebrate models [12] where the expression of SIX6 began later in development, around day 12 of differentiation (Figure 2.6B,D). After that time, SIX6 expression was maintained at high levels in the retinal spheres exclusively. Also, previously documented microarray data depicts a 40 fold increase

of *SIX6* in the retinal spheres when compared to the non-retinal spheres in culture, thereby validating our data further [117]. Hence, our data emphasizes that *SIX6* plays an essential role in retinal development.

An interesting feature about the expression pattern of *SIX6* is that it peaked at the same time that RAX expression decreased in these cells, at approximately day 10-day12 of differentiation (Figure 2.6C,D). This is interesting as the expression patterns of *RAX* and *SIX6* in humans have not been documented previously. Recently, a study speculated the role of *Six6* as a repressor of non-retinal genes in *Drosophila* [159]. Also, *Six3* and *Six6* have been known to interact with repressors like *groucho* to modulate eye development in *Drosophila* [160]. In vertebrate systems like medaka fish, *Six6* seems to play an important role for correct differentiation of amacrine cells and photoreceptors of the retina [161]. In our hiPSC-based system, the patterns of expression of *RAX* and *SIX6* indicate a potential relationship in which *RAX* may 'prime' the cells for the acquisition of a retinal fate whereas the expression of *SIX6* may function to commit the cells to a retinal fate.

In all, studies related to the transcriptional regulation of human retinal development have been limited. With the advent of hiPSCs, we have been able to investigate the roles of these factors in retinogenesis. Our data indicates the possible roles of *RAX* and *SIX6* to function as candidate transcription factors for specifying a retinal fate. However, future studies are needed to further investigate the exact role of *RAX* and *SIX6* in specifying a retinal fate in human development.

4.1 hiPSCs and Translational medicine

Beyond studies of human development, hiPSCs have also been implicated to play an important role in the field of translational medicine. They are pluripotent stem cells,

meaning that not only do they possess the capability to self-renew but can also differentiate into multiple cells types. Also, these have been derived from somatic cells with important implications for patient-specific medicine. Due to these properties, hiPSCs have been sought after in the field of regenerative medicine. hiPSCs have been implicated to play a role in the following avenues: Cell replacement, disease modeling, generation of patient-specific medicine and drug screening and development (Figure 4.3).

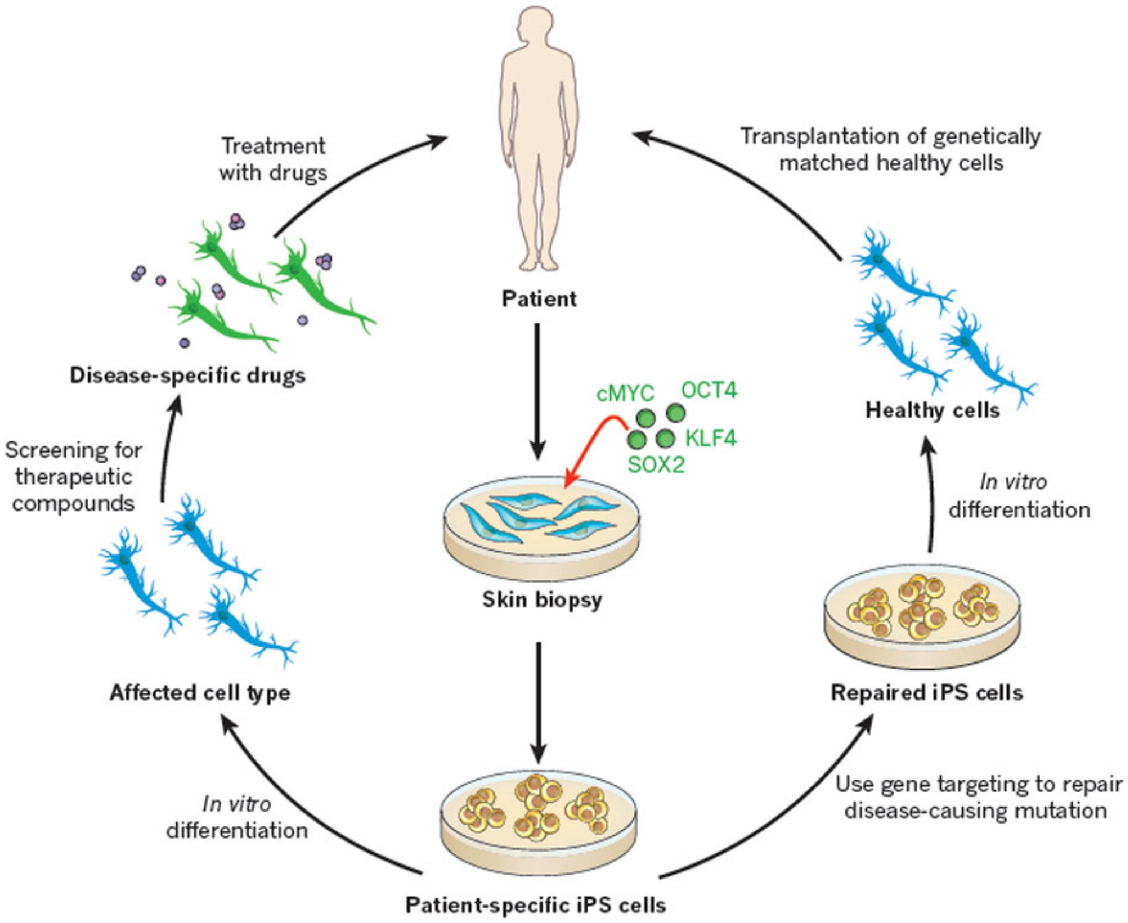

Figure 4.3. hiPSCs have numerous applications in translational research and therapy [45].

Although hiPSCs demonstrate a great potential for a wide range of translational applications, there have been some limitations to the application of this technology [162]. One of the main limitation stems from the methods used to derive these cells. The method used to reprogram the cells makes an important difference for future applications of hiPSCs. Traditionally, hiPSCs were derived using retroviruses or lentiviruses [53, 54]. However, the use of viruses as vectors opens up the possibility of their subsequent integration in the genome. Hence, vector systems employing episomal vectors [56], small molecules [163], vector-less, direct delivery of reprogramming proteins [163–165], as well as mRNA based reprogramming methods are currently replacing the traditional methods of reprogramming.

The next hurdle is the actual reprogramming event. The reprogramming efficiency of the non-viral methods are 1000 fold lower than those by viral methods [55], which had already been exceeding low (approximately 0.1% efficiency). Also, in some cases, incomplete reprogramming could lead to formation of aberrant stem cells with limited capacity to self-renew or differentiate [166]. The external environment also plays a key role in the reprogramming cells, as it has been demonstrated that cells respond best to conditions of hypoxia [167] or cultures supplemented with Vitamin C [168]. Even the diet of the donor can induce epigenetic changes in the resulting iPS cells [169]. Also, iPS cells have a high degree of variability, where some cell lines are more potent than others [170].

After successful generation and characterization of cell lines, the next hurdle is the actual transplantation event. iPS cells are highly variable and differ in their ability to form teratomas upon transplantation [171]. Also, insufficient amount of cells for transplantation, the technical difficulties of imaging the grafted cells, cell instability, side effects such as graft rejection and lack of humanized animal models further add to the reduced successful rate of cell replacement using hiPSCs [172].

It is thought that the use of non-xenogeneic components can help reduce rejection of the transplanted hiPSCs by the host [112]. In order to do so, hiPSCs must be maintained and differentiated in a non-xenogeneic environment with no animal components or serum. However, most methods of maintenance of hESCs and hiPSCs involve their growth on Mouse Embryonic Fibroblasts (MEF) as feeder cells or the use of matrigel, which is the extracellular matrix derived from mouse carcinomas [111]. Also, most differentiation protocols involve the use of FBS or KOSR in their differentiation protocol [114]. The use of such components can introduce unwanted xenogeneic components, leading to increased chances of rejection of hiPSCs upon transplantation. As an effort to remove all traces of animal components from hiPSCs, efforts have been made to made to maintain and differentiate hiPSCs in xeno-free conditions [173]. However, most studies lack in their ability to maintain a non-xenogeneic component throughout the course of differentiation of hPSCs [134].

The studies presented here help to overcome some of the challenges associated with the culture of hiPSCs under non-xenogenic components. First, the use of synthetically-coated plates eliminates the use of MEF or matrigel for efficient culture of hiPSCs. Second, the use of non-xenogeneic medium eliminates the use of animal components or serum in the medium, which is an improvement over the traditional methods. Hence, a non-xenogeneic environment was maintained by ensuring that all components used at every stage of culture of hiPSCs were devoid of any animal component or serum.

Beyond the growth of hiPSCs under non-xenogeneic conditions, it remained to be demonstrated that these cells could be effectively differentiated to a retinal fate. Thus, the cells were directed to differentiate with appropriate modifications to protocols previously established within the Meyer lab. Cells grown in a non-xenogeneic environment expressed an similarl compliment of transcription factors when compared to the hiPSCs grown on MEF and feeder-free systems (Figure 3.2A-H). At day 10 of differentiation, they acquired makers of the anterior neuroepithelium, with *PAX6*

being the main initiator of this stage [21]. Additionally, a full set of EFTFs were also expressed at this stage (Figure 3.2H). Interestingly, immature neural rosettes were also seen at this stage, which stained positive for *SOX1* (Figure 3.1A). The absence of mid brain and hind brain markers at this stage further confirms the anterior neural identity of these cells (Figure 3.2H).

At day 20 of differentiation, the cells organized themselves into two populations; retinal progenitor cells and non-retinal cells (Figure 3.3A). The retinal progenitors were identified by a lighter ring on the outside while the non-retinal cells lacked that morphological feature, as had been previously demonstrated for retinal differentiation under transitional systems [117]. Additionally, hiPSCs grown in the xeno-free medium gave rise to a slightly reduced number of retinal progenitors (about 15% retinal progenitors from a mixed population) from a mixed population when compared to MEF and feeder-free systems (20% efficiency) [93]. However, after this stage, almost all retinal progenitors developed into mature retinal cells, irrespective of the system they were grown in.

Further differentiation of hiPSC-derived retinal progenitors cells led to the expression of photoreceptor precursor markers like *CRX* at 50 days of differentiation. Cells reminiscent of the photoreceptor morphology were present in all three systems analyzed. Also, hiPSCs grown in non-xenogeneic conditions produced RPE robustly, with cells that became deeply pigmented and hexagonal in shape and expressed a full set of transcription factors specific to the RPE. This is particularly interesting as current efforts in the iPS field have been related to transplantation of RPE [104, 119, 174]. As our protocol highlights an efficient method to derive RPE spontaneously in a xeno-free environment, these cells can be further used for generating RPE cells for transplantation.

Previous efforts to derive RPE in a non-xenogeneic environment used FBS in their protocol, raising doubts of the true non-xenogeneic potential of these cells [124]. This is the first demonstrations of deriving retinal cells from hiPSCs under a xeno-free environment where care has been taken to ensure that all products are non-xenogeneic. Previous studies have generated clinical grade cell lines from hESCs [131]. These lines could be then be used to derive retinal cells using our protocol in future.

In summary, hiPSCs provide a unique model to recapitulate retinogenesis *in vitro* and function as a valuable tool for studies related to human development. Human iPS cells can be effectively specified towards a retinal lineage under conditions that underscore the applicability of hiPS cells for potential translational applications. Also, human iPS cell-derived retinal populations have the potential to be used in many applications, including transplantation, drug screening, and as a model for human retinal development and disease. In future, hiPSCs can be directed to generate more retinal cells *in vitro* to warrant effective cell replacement in diseases that affect the retina, including glaucoma and age-related macular degeneration.

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